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Studies on the Enzymatic System of Tumor Glycolysis.[1] I. Glycolysis of Free Sugar in Homogenates and Extracts of Transplanted Rat Sarcoma

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INTRODUCTION

Most of the intermediary reactions of the glycolytic cycle can easily be shown to take place in extracts of those mammalian tissues which form lactic acid anaerobically in the form of tissue slices. However, a steady formation of lactic acid from glucose, at a rate equal to or greater than that of the living or surviving tissue, is obtained only in special instances and with "fortified" systems. For example, the great difference encountered in muscle extract between glycogen and glucose metabolism can be traced to the lability of the muscle hexokinase. This enzyme catalyzes the reaction:

$$\text{glucose} + \text{ATP}^{2} \rightarrow \text{glucose-6-phosphate} + \text{ADP}.$$

If a stable hexokinase from yeast is added, glucose is easily transformed into lactic acid (1).

Brain hexokinase, on the other hand, is relatively stable, but because of the nucleotidase and ATPase present, small amounts of ATP,

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[1] This work was supported by grants from the American Cancer Society (recommended by the Committee on Growth), the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and the David, Josephine and Winfield Baird Foundation.

[2] Abbreviations used: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; ATPase = enzyme splitting the first labile group of ATP; apyrase = adenylpyrophosphatase, the enzyme splitting both labile P groups; pyro P = difference between 7 min. P and direct P; HDP = hexosediphosphate; HMP = hexosemonophosphate; PC = phosphocreatine; DPN = diphosphopyridinenucleotide or pure cozymase.

cozymase (DPN), HDP, and nicotinamide must be added to the extract. Moreover, the high rate of glycolysis encountered here depends, in the absence of phosphate donors, on the removal of the greater part of the ATPase or "apyrase" which is bound to the cell structure and removed by centrifugation (3,4).

Tumor homogenates and extracts offer striking examples of the fact that a continuous rapid glycolysis is the result of a balanced activity of the two enzymes, hexokinase and ATPase. Moreover, tumor hexokinase shows different affinities for glucose and fructose at a higher range of ATP concentrations than was found with extracts from brain. On the other hand, dissolved tumor ATPase exhibits a special sensitivity to some narcotics which is not encountered with ATPase of normal tissues or with other types of this enzyme which we have tested.

It will be shown in the following pages that these features of the glycolytic system of malignant tumor enable the investigator to obtain by specific procedures, in homogenates and extracts, a steady and very high metabolism of glucose in the absence of phosphate donors. The metabolic rates of different sugars then closely resemble those of the tumor cell.

METHODS AND PROCEDURES

The tumors were kindly supplied by Dr. Margaret Lewis of the Wistar Institute.[3] Generally, rat sarcomas No. 304 and 231, originally induced by methylcholanthrene, were used. We removed the tumors during the period of rapid growth—about 12 days after transplantation. In the present paper we report the results with transplanted rat sarcoma. In the paper which follows a comparison is made with several types of mouse tumor. Necrotic and hemorrhagic parts were removed before homogenization.

The general procedure was the same as that used with brain (2). Homogenates were prepared according to Potter and Elvehjem (5), and used directly or after centrifuging for 6 min. at 4000 r.p.m. in the refrigerated centrifuge. Lactic acid was determined manometrically. Occasionally, the results were checked by chemical determinations, using the Lieb and Zacherl apparatus (6). The CO_2 evolved by the splitting of the added ATP (50–90 γ 7 min. P/sample) amounting to 35–60 mm.^{3} *in maximo*, was determined in the blank, where very little lactic acid is formed, but we refrained from correcting the manometric figures accordingly.

The following preparations were used: ATP prepared according to Kerr, as modified by Polis and Meyerhof (7); HDP purified from commercial calcium or barium salts (Schwarz, Inc., N. Y.) according to Meyerhof and Junowicz-Kocholaty (8); HMP prepared from maceration juice of yeast (Robison ester) (9); cozymase, 65% pure DPN, commercial preparation from Schwarz, Inc., N. Y. Yeast hexokinase was prepared according to Meyerhof (1) and purified as described by Berger *et al.* (10).

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[3] We are indebted to Dr. M. Lewis for this continuous help.

Phosphocreatine was prepared in this laboratory as in (4). Adenosine-5-monophosphoric acid was a crystallized preparation from Laokoon, Lwow, Poland. The sugars used were commercial preparations of the highest purity obtainable.

The homogenates were made with 4 parts of isotonic solution. This solution contained: 62 parts of 0.9% NaCl, 3 parts of 1.15% KCl, 3 parts of 7.8% $MgSO_4$ $(+7H_2O)$, 16 parts of 1.3% $NaHCO_3$, 16 parts of 1% NH_4HCO_3; 2% of nicotinamide was dissolved in this mixture.

For the manometric experiments the complete system usually consisted of the following components in a total of 1.3–1.8 cc. (38°C.): 0.3–0.5 cc. homogenate or centrifuged extract in the isotonic solution given above, 0.1 cc. phosphate $M/10$ (pH 7.4), 0.1 cc. 1.3% $NaHCO_3$, 0.1 cc. $M/10$ Na pyruvate, 0.05 c.c 7.8% $MgSO_4$ $(+7H_2O)$, 0.05 cc. HDP containing 16 γ P (equal to 0.5 μM bound phosphate or 11 $mm.^3$ CO_2), 0.2–0.5 cc. Ringer solution, or various sugars, inhibitors, *etc.*, depending on the special purpose of the experiment.

After temperature equilibrium was established, 0.1–0.15 cc. ATP containing 50–90 γ Pyro P, 0.15 cc. cozymase, containing 0.5 mg. DPN, and 0.1 cc. 1.3% $NaHCO_3$ were added from the side arm.

The selection of the components and accessory substances of this solution was made on the basis of our former experience with brain homogenates and those of other authors with similar preparations. Pyruvate, which has no effect on brain homogenate, shows a very marked increase of lactic acid formation in tumor homogenate (see also 11). In this case also there is a close agreement between the manometric and chemical determination of lactic acid. But some of this lactic acid is now formed by dismutation between pyruvate and triosephosphate, while a corresponding amount of phosphoglyceric acid derived from sugar accumulates, due to damage of the enzymes which transform the 3-phosphoglyceric into pyruvic acid

To measure the turnover of phosphate in the absence of the oxidation-reduction step of cozymase, the system was simplified. The solution consisted only of NaCl-KCl with $MgSO_4$ and $NaHCO_3$. The activity of hexokinase was usually determined in the presence of 8×10^{-2} M NaF, which inhibits the ATPase about 70%. The activity of ATPase was determined in the presence and absence of NaF. The determinations of free sugars were done according to the method of Nelson (12).

Former experiments in the Heidelberg laboratory had indicated that K or NH_4 favor transphosphorylations (13). Guided by this experience, we replaced the $NaHCO_3$ by a mixture of $NaHCO_3$ and NH_4HCO_3 for the tumor homogenate and obtained decidedly increased glycolytic rates. Racker and Krimsky (14), as well as Novikoff *et al.* (11), avoided Na completely and replaced it by K in every component of the glycolytic mixture. In several trials with this system we have not found any systematic differences from the mixture used by us. Nevertheless, it is true that K or NH_4 in relatively high concentrations are necessary for maximal rates of glycolysis, while Na seems unnecessary, and even harmful, unless compensated by the other ions.

RESULTS

A. Fresh Tumor

No matter what kind of tumor is used, the results with homogenates and extracts of fresh tumor (unfrozen) are very similar: HDP gives a continuous glycolysis with a Q_{La} (mm.^{3} CO_2 driven out by lactic acid in 1 hr. for 1 mg. dry weight of original tumor tissue) of 30–50. HMP (Robison ester) shows for the first 20–30 min. a very high rate, which, calculated for 30 min., corresponds to about 80, but whose computation is rather arbitrary because of its rapid decline and its dependence on the concentration. Glucose and other free sugars produce either the same CO_2 as the blank, which corresponds—in addition to the small amount derived mainly from HDP—to the splitting of the added ATP, or they show some additional glycolysis, during the first 10–20 minutes, with a rapid decline afterward.

Chemical determinations confirm this result. In this case it is not necessary to incubate the extract at 38°C. before adding the coenzymes; however, contrary to expectation this does not improve the yield of lactic acid. Only when phosphate donors are added, such as HDP or phosphocreatine (PC), is the lactic acid formation increased. In the case of HDP, most of the lactic acid comes from the HDP itself; the experiments with phosphocreatine, therefore, seem more interesting. (Because of the alkalinization which accompanies the splitting of PC, only chemical determinations have been made.)

Example. (No. 333) 0.5 cc. centrifuged extract (16 mg. dry weight of tumor) in 2 cc. gives in 20 min. with the incubation mixture:

without sugar, but with 1 μM of HDP (Blank)	0.30 mg. lactic acid
as above, with 4 mg. glucose	0.61 mg. lactic acid
as above, with 4 mg. glucose + PC (5.7×10^{-3} M)	0.99 mg. lactic acid
as above, with HMP containing 3 mg. hexose	1.21 mg. lactic acid

In the presence of NaF, where all lactic acid is derived from pyruvate, while an equivalent amount of phosphoglyceric acid is formed, the following values were obtained:

with 1 μM of HDP alone	0.31 mg. lactic acid
with 4 mg. glucose	0.55 mg. lactic aid
with 4 mg. glucose + PC	0.73 mg. lactic acid
with HMP containing 3 mg. hexose	0.835 mg. lactic acid

Further experiments of this type showed that, even in the presence of phosphate donors, the lactic acid formation from glucose declined quickly.

Because of our previous experience with brain homogenates and extracts, this unsatisfactory result, as regards steady glycolysis of free sugar, was interpreted as due to a relative lack of hexokinase compared with the amount of ATPase. In this way, ATP would be destroyed more quickly than it is regenerated and the glycolytic cycle would get out of step. This assumption has been strikingly confirmed.

B. Activity of Hexokinase and ATPase

Homogenates, as well as centrifuged extracts, contain so much hexokinase that about 30 γ pyro P of ATP can be transphosphorylated to glucose by 0.5 cc. extract (15 mg. of tissue dry weight) in 5 min. Table I gives several such experiments in the presence and absence of NaF and with different concentrations of glucose and fructose. The difference in transphosphorylation will be discussed in section G. On the other hand, the homogenate contains so much ATPase that in 5 min. 240 γ pyro P of ATP are dephosphorylated. The extract, after 5 min. centrifugation at 4000 r.p.m., still dephosphorylates 150 γ pyro P of ATP in 5 min. This is in striking contrast to brain homogenate, where 9/10 of the ATPase is bound to the structures and removed by centrifugation. Although, with 10 min. centrifugation more ATPase is removed, other glycolytic enzymes are removed at the same time. The relative rates of splitting of ATP are shown in Fig. 1.

The disproportion between hexokinase and ATPase can be abolished by two means: either by adding more hexokinase, or by inhibiting the ATPase without altering the amount of the other enzymes of the glycolytic cycle. Both procedures give the same result, *i.e.*, they produce a high and steady glycolysis of free sugar for 60–90 min. in the absence of phosphate donors.

TABLE I

Activity of Hexokinase of Tumor Extract; ATP Turnover with 0.5 cc. Extract, at 38°C.

No.	Total vol.	Time	Conc. NaF	Sugar	Hexose	Δ Inorg P split off	Pyro P present	Δ 7′ P
	cc.	*min.*	(*M*)		*mg.*	γ	γ	γ
400	1.2	0	—	—		—	131	—
		20		—		45.6	78.5	− 6.4
		5		Glucose	2	14.6	90	−25.9
		20		Glucose	2	22.8	58.1	−49
		5		Fructose	2	16.3	91	−21.8
		20		Fructose	2	29.6	60	−40.3
407	1.2	0	8×10^{-2}	-		—	113	—
		10	8×10^{-2}	—		47	61.5	− 4.6
		10	8×10^{-2}	Glucose	2	24.2	58	−31.0
		10	8×10^{-2}	Fructose	2	31.4	66	−15.5
407	1.2	20+0[a]	8×10^{-2}	—		—	117	—
		20+10[a]	8×10^{-2}	—		48.6	58	−10
		20+10[a]	8×10^{-2}	Glucose	2	34.2	57	−25.8
440	1.0[b]	0	1×10^{-1}	—		—	179	—
		10	1×10^{-1}	—		33.6	118	−27
		5	1×10^{-1}	Glucose	2	15.3	99.5	−64.3
		10	1×10^{-1}	Glucose	2	18.3	85	−75.7
		5	1×10^{-1}	Fructose	2	19.2	102	−51.2
		10	1×10^{-1}	Fructose	2	24.3	95.5	−59.5
		5	1×10^{-1}	Fructose	20	12.9	102	−64.3
		10	1×10^{-1}	Fructose	20	15.3	83.5	−80.5
463	1.4[c]	0	7×10^{-2}	—		—	127	
		8	7×10^{-2}	—		47	80	0
		8	7×10^{-2}	Glucose	1.4	26	59	−42.0
		8	7×10^{-2}	Fructose	1.4	39	72.6	−17.0

[a] Incubation for 20 min. before addition of ATP.

[b] Sarcoma homogenate, with satd. octyl alcohol.

[c] 1.4×10^{-2} *M* azide.

C. Addition of Yeast Hexokinase

If purified yeast hexokinase is added to the glycolytic system, glucose and fructose are as quickly metabolized as HMP during the initial period, and, under suitable conditions, the rate is maintained for

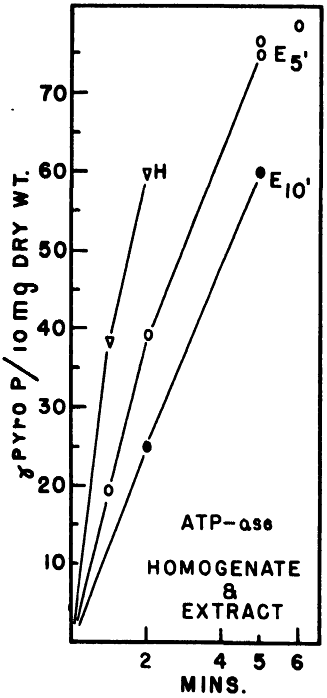

Fig. 1. Activity of ATPase in homogenate (H) and extract (E) of rat sarcoma (Exp. 310) $E_{5'}$ = extract, 5 min. centrifuged at 4000 r.p.m. $E_{10'}$ = 10 min. centrifuged at the same speed. Ordinate = γ pyro P split off from ATP at 38°C. for 10 mg. dry weight of tumor tissue from which extracts or homogenates are made. The circles of $E_{5'}$ at 5 and 6 min. correspond to different experiments.

about 1 hr. Q_{La} values between 60 and 90 are obtained in this way, even in the absence of pyruvate (Tables II and III). This result proves that all other enzymes are present in sufficient amounts and only hexokinase is deficient. As can be seen from Fig. 2, the rate of glucose in the presence of yeast hexokinase is, for the first 20 min., about the same as that of HMP in the absence of hexokinase, but remains steadier. This must be attributed to the continuous production of HMP. This reaction therefore, controls the speed.

A comparison of manometric and chemical determinations of the same experiment (No. 426) is given in Table III. Generally the Q_{La} values are calculated without any correction for the increase in acidity due to dephosphorylation of ATP. If such a correction is applied, as in the penultimate column, assuming that all added ATP is de- or transphosphorylated in 1 hr., the corrected value becomes somewhat too small. Although the agreement of the manometric and chemical determinations is, therefore, never very close, it is still rather good.

TABLE II

Q_{La} Values of Tumor Extract in Presence and Absence of Yeast Hexokinase

No.	Extract		Sugar	Hexose	Dilution of stock hexokinase	Q_{La} for first 30′	Q_{La} for 60′
	cc.	Tumor					
				mg.			
390	0.3	Fresh	—		—		8.5
			Glucose		—	16	11.5
			Glucose	4	1:10	108	57
			Glucose	4	1:30	100	80
			HMP	3	—	78	53
408	0.5	Frozen one day	—		—		8.5
			Glucose	3	—	30.6	24.5
			Glucose	3	1:15	83	58
			HMP	4	—	69	48
424	0.5	Fresh	—		—		10
			Glucose	4	—	21	13.5
			Glucose	4	1:10	115	78
			Glucose	4	1:25	104	84
			HMP	4	—	78	52
426	0.5	Fresh	—		—		9.5
			Glucose	4	—	22	13.5
			Glucose	4	1:9	88	59
			Glucose[a]	4	1:9	90	60
			HMP	4	—	54	38

[a] No pyruvate

TABLE III

Chemical and Manometric Determination of Lactic Acid

0.5 cc. tumor extract, 1 hr., 38°C., No. 426.

Additions	Manom. CO_2	Chem. l. a. formed	Chem. l.a. equivalent to CO_2	Q_{La} manom.	Q_{La} manom. (corr. for ATP split)	Q_{La} chem. calc.
	mm³.	*γ*	*mm³.*			
—	127	333	83.5	9.5	2.5	5.8
HMP	505	2190	520	38	31	38
Glucose + yeast hexokinase	830	2590	675	59	52	47
Glucose + yeast hexokinase (no pyruvate)	840	3060	767	60	53	53

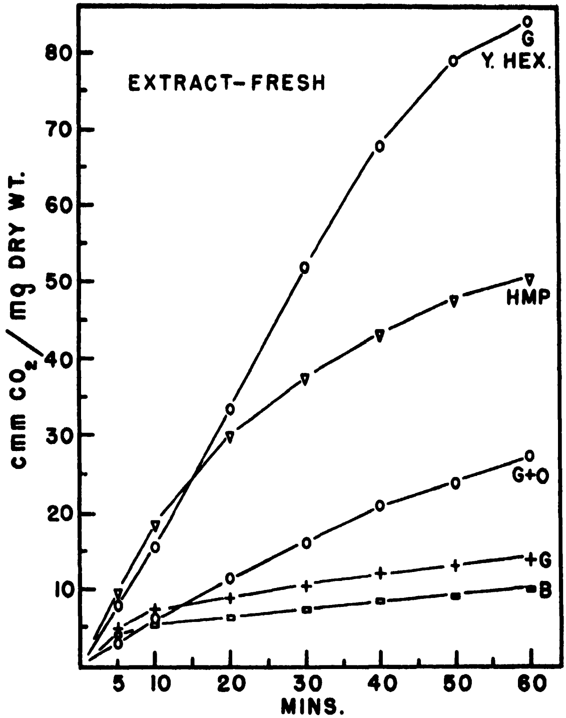

Fig. 2. Glycolysis in the extract of fresh rat sarcoma (Exp. 424). Ordinate = mm^3. CO$_2$/mg. dry weight of tissue. Total vol. = 1.8 cc. Each sample contains the complete incubation mixture unless otherwise stated. B = blank. G = 4 mg. glucose. G + O = glucose with octyl alcohol G, Y. Hex. = glucose with diluted yeast hexokinase. HMP = Robison ester with 4 mg. hexose.

D. Glycolysis in Extracts from Frozen Tumors

Without having recourse to the introduction of a foreign enzyme, the hexokinase and ATPase of tumor can be brought into step by weakening the latter without damaging the other enzymes of the glycolytic cycle. To accomplish this, 2 procedures have proved successful, although in different degrees.

One consists in freezing the tumor. If the tumor is frozen for only 2–3 hr. in the freezing compartment of a refrigerator, the activity is not appreciably changed. But if it is frozen for 1–3 days, glycolysis of glucose starts at a high rate after addition of the coenzymes. This high rate is maintained generaly for only 20–30 min., but occasionally for over an hour. In Fig. 3 two examples are given for the result of one day's freezing, one for the homogenate and one for the extract. Curve H* (homogenate) shows a strong increase over the curve of the unfrozen tumor (H) of an experiment of the day before, for the first 20 min. only. The dotted line E* referring to a similar experiment made with centrifuged extract, shows, on the other hand, a glycolytic rate which actually increases somewhat for a period of 1 hr. or longer, with a Q_{La} of about 40. The blank value is given by the dotted line BE, and the glycolysis of fructose in FE*. Generally, the glycolytic rates of

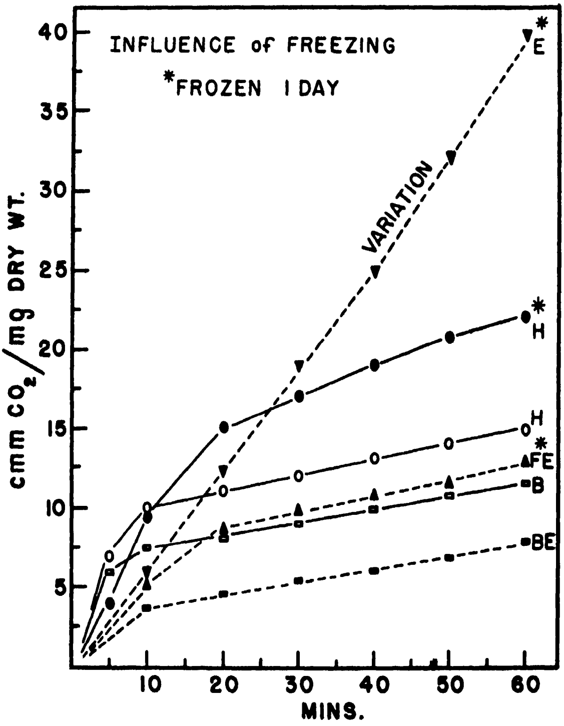

FIG. 3. Glycolysis in homogenates and extracts of frozen rat sarcoma. Continuous line = experiments with homogenates (444, 445); dotted lines = experiments with extracts (426, 427). B = blank for homogenate of fresh and frozen tumor. H = glucose in fresh homogenate. H* = the same for tumor frozen one day. B.E. = blank for fresh and frozen extract. E* = glucose in extract of tumor, one day frozen. FE* = fructose (0.25%) in the same extract. The slightly increasing rate in E* is a variation of the norm because most curves for extracts of frozen tumors would bend downwards after 30–40 min.

glucose in frozen extracts remain high for 30–40 min. before falling off, while those of homogenates fall off somewhat earlier. But a rapid constant rate for over an hour is rather exceptional. In this experiment the activity of ATPase was tested the next day and found to be unusually low: in 10 min. in the presence of NaF no ATP was split by 0.3 cc. of extract (9 mg. dry weight of tumor).

On the other hand, in cases like those of curve H*, Fig. 3, no definite decrease of the rate of dephosphorylation of ATP in the presence of NaF was visible after 1–3 days' freezing. Nevertheless, it is probable that in the complete system it would show some decreased reactivity. Whatever may be the full explanation for this partial restoration of the glycolysis of free sugar, a completely clear picture is obtained from the experiments described in the next section.

E. Effect of Higher Members of Narcotic Series

A continuous glycolysis of free sugars for at least 1 hr. can be obtained with homogenate or centrifuged extract from fresh or frozen tumor if octyl, decyl, or lauryl alcohols, or toluene are added to saturation. Because toluene has a high vapor pressure, and special precautions are necessary to avoid errors in the manometric readings, most of the experiments were done with octyl alcohol. With frozen tumor the Q_{La} values are generally higher.

The reason for this paradoxical influence of the narcotics is the strong inhibition of the ATPase of tumor extract (see Fig. 4). Thus far we have not found an ATPase from other cells which is inhibited in this way. Dissolved ATPase from muscle and from yeast are not influenced.[4] ATPase of brain, adsorbed on the washed particles (5) or in homogenates, is likewise unaffected, but in the extract it is activated about 100% during the first 5 min. of incubation. The activation disappears in the following minutes (Table IV). In homogenates of liver and kidney, ATPase is slightly activated, while in extracts it is unaffected or somewhat inhibited.

In homogenates and extracts of tumor tissues the inhibition of ATPase by octyl alcohol or toluene amounts to 45–60% in the first 5 min. and increases progressively. If the extract is incubated with octyl alcohol for 30 min. at 38°C., the ATPase activity afterwards is

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[4] Adsorbed ATPase of dry yeast, however, is strongly inhibited, as will be shown in a later communication.

TABLE IV

Influence of Octyl Alcohol on Crude ATPase of Different organs ATP split at 38°C.

No.	Organ	Preparation	Amount	Time	ATP split		Change
					Control γ P	Octyl γ P	
			cc	*min.*			*per cent*
	Muscle (rat)	Mg ATPase	0.2	5	32.1	30.3	—
425	Brain (rat)	Extract+NaF	0.5	3	24	45.5	+90
	Brain (rat)	Extract+NaF	0.5	3–6	10.6	12.3	+18
412	Brain (rat)	Extract	0.3	10	18.6	24.5	+30
420	Brain (rat)	Homogenate+NaF	0.3	4	44.3	47.9	+ 8
464	Liver (rat)	Extract	0.5	6	78.3	80.3	—
465	Liver (rat)	Extract+NaF	0.5	10	72.5	64.2	−11
	Liver (rat)	Homogenate+NaF	0.5	5	72.3	88.5	+22
466	Kidney (rat)	Extract+NaF	0.5	10	73	50	−30
	Kidney (rat)	Homogenate+NaF	0.5	5	49	57.5	+18
442	Yeast[a]	Purified ultrasonic solution	0.3	15	70.0	68	—
	Yeast[a]	Purified ultrasonic solution	0.3	15–30	21.9	20.7	—
429	Rat sarcoma	Extract	0.3	6	67	36	−46
	Rat sarcoma	Extract+NaF	0.3	6	45.8	19	−58
421	Rat sarcoma	Extract+NaF	0.3	6	40.3	18.5	−59
	Rat sarcoma	Extract+NaF	0.3	6–12	29.2	2.0	−93
424	Rat sarcoma	Homogenate	0.3	2	51.7	26.8	−48
440	Rat sarcoma	Homogenate+NaF	0.3	10	71.0	33.3	−53
443	Mouse carcinoma	Homogenate	0.3	2	69	42.2	−40
449	Mouse sarcoma	Extract (frozen)	0.2	3	13.5	4.2	−69

[a] 30°C.

inhibited 75%. This corresponds to the situation prevailing in the manometric experiments, because the narcotic is added to the extract from the beginning, and about 30 min. elapse before the glycolysis starts with the tipping in of the coenzymes. Decyl and lauryl alcohols have, in the first 20 min., a much weaker influence than octyl alcohol. However, the inhibition is likewise progressive. Incubation with saturated decyl alcohol for 30 min. gives a subsequent inhibition of ATPase of 50–60%, which explains the effect on glycolysis. Heptyl alcohol gives a lower rate of glycolysis which, however, is steady similarly to the other higher alcohols. Probably it inhibits some other intermediary enzymes besides ATPase.

The hexokinase of tumor is apparently not influenced by narcotics; transphosphorylation is either unchanged or somewhat increased. This

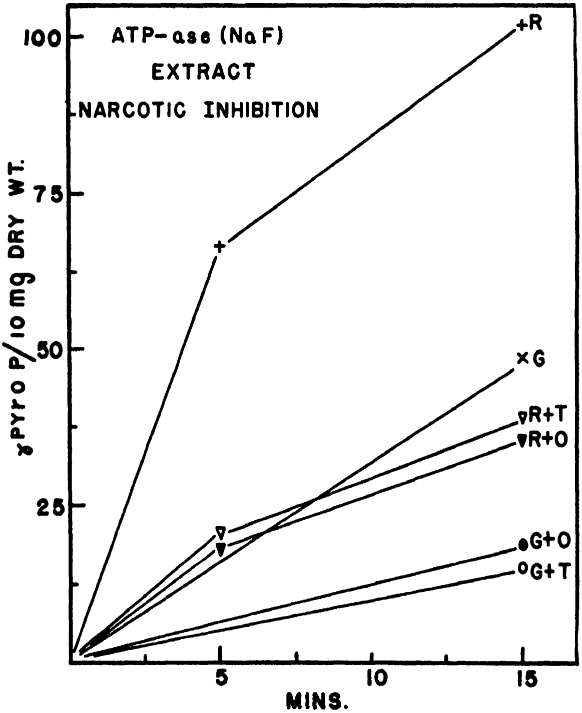

Fig. 4. Activity of ATPase in the presence of NaF (8×10^{-2} *M*). Influence of narcotics. R = no addition. R + T = with saturated toluene. R + O = with saturated octyl alcohol. G = with 4 mg. glucose. G + T = with glucose plus saturated toluene. G + O = with glucose plus saturated octyl alcohol.

latter result is probably caused indirectly by the preservation of ATP due to the inhibition of the ATPase. It may also be noted that transphosphorylation with glucose strongly decreases the ATPase activity because most of the ATP is removed from the sphere of action (see Fig. 4, curve G).

The breakdown of HMP depends only on phosphohexokinase which is present in excess of the hexokinase, as can be seen by the experiments with addition of yeast hexokinase. At the same time, HMP needs more ATPase in the steady state than glucose, because it functions also as P donor. Consequently, HMP turnover is inhibited about 30% by octyl alcohol in the rapid period [see also (4)].

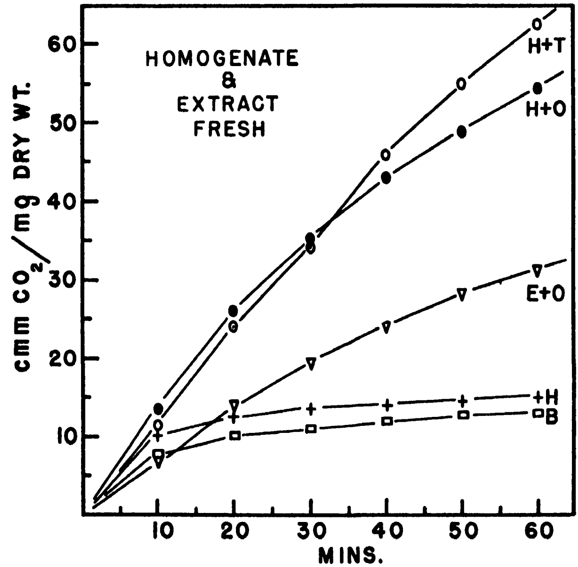

FIG. 5. Glucose metabolism with toluene and octyl alcohol (No. 437). B = blank without narcotic. H = glucose metabolism in homogenate without narcotic. H + T = the same with toluene. H + O = the same with octyl alcohol. E + O = glucose metabolism in the extract from the same homogenate plus octyl alcohol.

Glycolysis in homogenates and extracts of fresh tumor with these narcotics is demonstrated in Fig. 5. Under these conditions, homogenates give higher values than extracts. Q_{La} with toluene is about 60 (H + T), with octyl alcohol (H + O) about 55, but in extract only 30 (E + O). Glucose without octyl alcohol (H) gives only a trifle more than the blank.

F. Effect of Sodium Azide

Some further substances were tested for inhibition of ATPase and for activation of glycolysis. Sodium azide in 0.005 *M* concentration inhibits the ATPase of sarcoma, brain, and some other organs about 40%. It elicits a strong glycolysis of glucose in the homogenate and extract of sarcoma, which usually lasts only 30 min., but sometimes lasts longer. With still higher concentrations of azide (0.012 *M*) the rate of glycolysis is quite steady for 1 hr. or more, with Q values of about 30 in centrifuged extracts. An experiment of this type is shown in Fig. 6 (No. 486) where, in the presence of azide (curve G + A), the rate of glycolysis in an extract of fresh rat sarcoma is slightly higher than with saturated lauryl or decyl alcohol (G + L & D, same points for both alcohols). This result must be compared with curve G of the same figure: glucose, without additions.

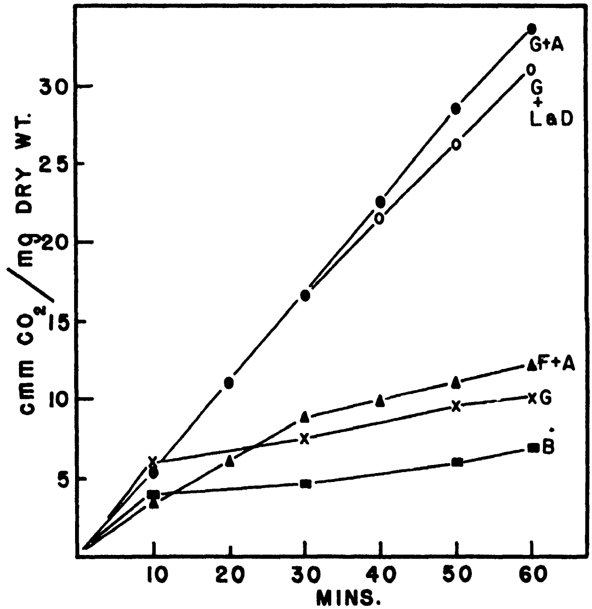

Fig. 6. Glycolysis in extract of fresh tumor in the presence of azide and of higher alcohols (Exp. 486). B = blank; G = 0.2% glucose without further addition; G + L & D = glucose with saturated lauryl alcohol and with saturated decyl alcohol. (The curves were identical.) G + A = 0.2% glucose in the presence of 0.012 *M* sodium azide; F + A = 0.2% fructose in the presence of 0.012 *M* sodium azide.

G. Metabolic Rates of Various Sugars

A comparison of the rates of glycolysis of various sugars and at different concentrations of sugars, shows that, by checking the excess of ATPase, we obtain a glycolysis closely resembling that of the living tumor tissue. A typical experiment is given for the homogenate of fresh tumor in Fig. 7. Fructose, 0.25%, gives a rate of glycolysis very little over the blank, but 2.5% fructose gives an appreciable reaction, ⅔ to ¾ that of 0.25% glucose. Glucose (2.5%) also gives a higher rate than 0.25%, but the increase amounts to only 20%. Mannose (M) gives nearly the same turnover rate as glucose. The curve for 2.5%

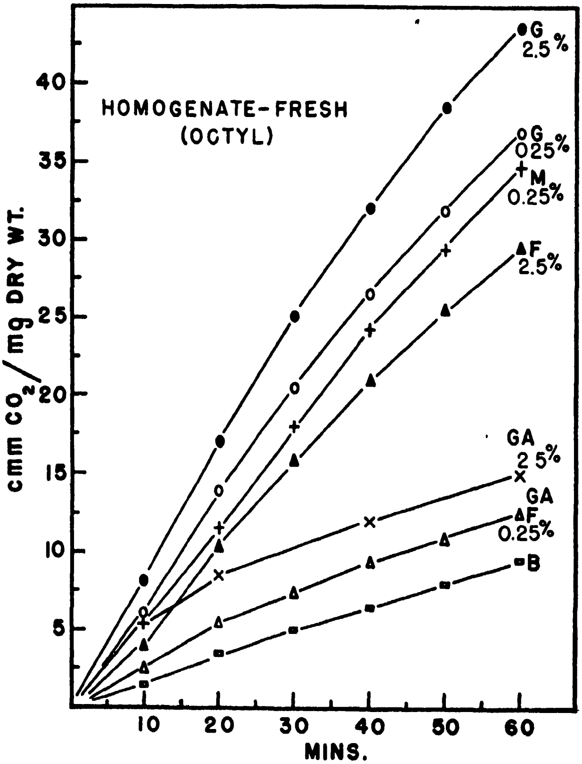

Fig. 7. Glycolysis of various sugars in the homogenate of fresh tumor in the presence of octyl alcohol (Exp. 445). B = blank; G = glucose; M = mannose; F = fructose; GA = galactose. The curve for 2.5% mannose, not drawn, nearly coincides with that of 2.5% sodium azide.

mannose is not drawn, but it would nearly coincide with that of 2.5% glucose (G 2.5%) in Fig. 7. On the other hand, dilute galactose (Ga) gives as low values as low concentrations of fructose, and higher concentrations give little more. This approaches the relation in tumor slices except that there concentrations of 0.2–0.25% are already optimal for glucose and mannose. An experiment with centrifuged extract is reporduced in Fig. 8. The outcome is similar, although the rate for 2.5% glucose (G 2.5%) is about ⅓ higher than for 0.25% glucose. With 2.5% fructose, the rate of breakdown is less than half of 2.5% glucose. Still, a very marked difference exists between high and low concentrations of fructose if the blank (B) is taken into account.

In brain *extract* it is found that a similar difference between glucose and fructose appears if the concentration of added ATP is very low, about a tenth of the concentration used here. In brain *homogenates*,

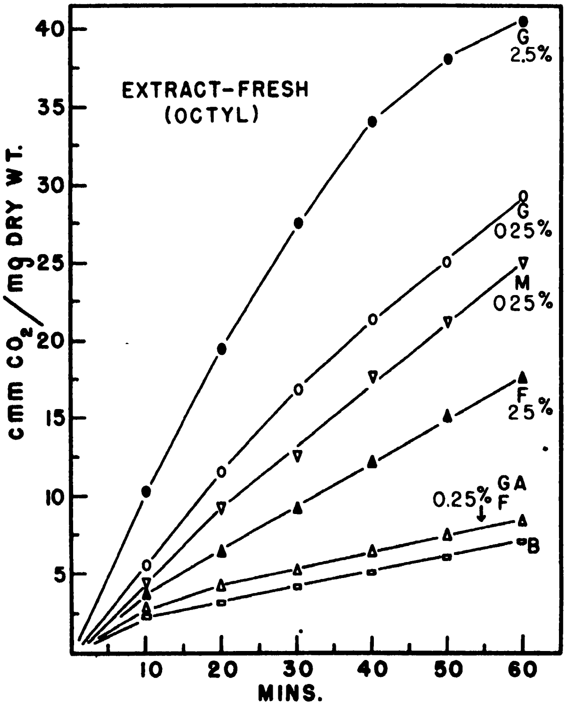

FIG. 8. Glycolysis of various sugars in extract of fresh tumor in the presence of octyl alcohol (Exp. 450). B = blank; G = glucose; M = mannose; F = fructose; GA = galactose. The curves for 0.25% fructose and 0.25% galactose coincide.

however, this difference, as well as a low rate of glycolysis, is conspicuous with relatively high concentrations of added ATP. This was attributed to the difference in the final content of ATP caused by the ATPase bound to the structures. Because the ATPase of tumor is much less firmly bound to the structures, we understand that the difference between homogenate and extract is small, and that both show the same features toward various sugars. Octyl alcohol diminishes, but does not abolish the activity of ATPase; the concentrations of ATP are therefore actually always lower than the added amounts.

To elucidate this point further, we determined in tumor extract and homogenate the hydrolysis curve of the organic phosphate present in the manometric experiments after 1 hr. of incubation; these showed that ATP had nearly disappeared in the absence of sugar, as well as in the presence of glucose without octyl alcohol, while with sugar + octyl alcohol $\frac{1}{3}$–$\frac{1}{4}$ of the initial amount was still present. We conclude, therefore, that the different affinity for glucose and fructose in sarcoma extract and homogenate occurs at relatively higher concentrations of ATP than in brain (around 3×10^{-4} *M*). Even in the transphosphorylation experiments with 2×10^{-3} *M* ATP, fructose reacts less. This is especially conspicuous with sugar concentrations as low as 0.1 and 0.05% (see Table I, No. 463 and the following paper, Table II, No. 462), while with 2% sugar the difference completely disappears (Table I, No. 440). Because the change of 7 min. P is no accurate measure of the phosphorylation of fructose, owing to a partial formation of easily hydrolyzable fructose-1-phosphate, the disappearance of free sugar was measured directly by the method of Nelson (12). With 0.05 and 0.1% fructose, hardly any sugar was esterified while the same concentrations of glucose showed as much esterification as 0.25% glucose.

It should be emphasized that this relative activity of the sugars is not connected with the action of the narcotics. They only allow one to demonstrate it clearly. In the experiments with frozen tumor or with sodium azide the same differences appear; in the experiment called "variation" in Fig. 3, a parallel run was made with fructose, which gave the curve EF*. See also curve F + A in Fig. 6.

Discussion

The present paper is concerned with the factors which enable homogenates and extracts of malignant tumors to glycolyze free sugars at a high, and more or less constant, rate. Such a glycolysis can always be obtained if enough phosphorylated hexoses are added, as was shown quite recently by Novikoff *et al.* (11).[5] In the absence of such phosphate donors, a steady turnover is only possible if the hexokinase and the ATPase or "apyrase" keep pace. This requires that the activity of the ATPase, in terms of phosphate turnover, be twice that of hexokinase. According to our scheme of glycolysis (see, *e.g.*, 15), one mole of HDP is formed by the consecutive action of hexokinase and phosphohexokinase, each transferring one mole of phosphate; these two moles of phosphate finally appear in two moles of phosphopyruvate. Two additional moles of phosphate are incorporated in one hexose unit as carboxylphosphates of 1,3-diphosphoglyceric acid. In the steady state two of these four energy-rich phosphates must be split by ATPase while two others are transferred to the next hexose unit, to undergo the same transformation.

As shown in section A, about 8 times as much ATPase as hexokinase (in terms of phosphate turnover) are present in the tumor homogenate, and about 6 times as much in the extract, with wide variations. If the ATPase were inhibited to $\frac{1}{4}$ or $\frac{1}{3}$ of the original activity, while the hexokinase and all other enzymes remained unaltered, then a completely constant rate of glycolysis would be obtained. This is, roughly, although not quite precisely, what happens in the presence of octyl alcohol or toluene. Because the inhibitions are progressive, and the other enzymes are slowly losing their activity, the steady state is maintained only for about 50-80 min. An additional complication is the presence in tumor homogenate of an adenosine-5-phosphatase with an absolute activity of about 30% of the ATPase, which destroys irreversibly the adenylic acid which may be formed. The activity of this enzyme is 10 times as high as that of the nonspecific phosphatases, which are present, too. Moreover, this enzyme is not inhibited by octyl alcohol.

The remarkable fact that octyl alcohol inhibits the ATPase in tumor extract much more than in extracts of normal organs may be compared with the fact that it causes a turbidity and a slow precipitation of protein in the former. Octyl alcohol and toluene generally inhibit only enzyme reactions on cell surfaces but not in solution (O. Warburg, 16). This rule has several exceptions (see 4). Such an exception is the

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[5] We are obliged to Dr. Novikoff for allowing us to read the manuscript of this paper before it appeared in print.

ATPase of malignant tumor which, in solution, behaves toward surface active substances as if it were an enzyme bound on the living interfaces of cells.

Because the relative turnover rates of various sugars, and of different concentrations of the same sugar, resemble those of the living tumor cell, we assume that we have reconstructed the situation of the glycolytic system *in vivo* fairly well by the artificial means of addition of narcotics to the homogenate. *In vivo* the excess of ATPase is probably separated by cell structures from its substrate, and the organization of the cell keeps the different enzymes in step. Whether the active concentration of ATP in the tumor cell is very much lower than the total concentration, 180 γ pyro P/g. wet weight, according to Lohmann (17), is not so easy to decide as for brain, because the difference between the rates for glucose and fructose appear in tumor homogenates with as high a concentration as 60 γ pyro P/cc.

SUMMARY

In homogenates and centrifuged extracts of malignant tumor, a steady and rapid glycolysis of free sugar in the absence of large amounts of phosphate donors is obtained if the activities of the hexokinase and ATPase are brought into step. This is made possible either by addition of yeast hexokinase or by inhibition of tumor ATPase, without altering the activity of the other enzymes concerned.

ATPase of tumor is weakened by long freezing of the tumors, but with greater regularity by the addition of some high members of the narcotic series; toluene, decyl and octyl alcohols are best suited. Other inhibitors of the ATPase, such as azide in high concentration (over 0.01 *M*), act similarly. The ATPase of normal tissues is only slightly affected by octyl alcohol.

With octyl alcohol Q_{La} values of 40–60 are obtained for glucose in tumor homogenates and lower values in extracts. The differences in the rates for various sugars, and for different concentrations of the same sugars, closely resemble those obtained for the living tissue in the absence of inhibitors.

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Studies on the Enzymatic System of Tumor Glycolysis.[1]
II. Comparative Study of Rat and Mouse Tumor Homogenates

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INTRODUCTION

It seemed of interest to investigate whether the extracts and homogenates of various malignant tumors of mice showed the same specific and balanced activities of the glycolytic enzymes as the transplanted rat sarcoma. For this comparison we had only a few types of transplanted mouse sarcomas and one carcinoma at our disposal. As will be shown below the observed regularities as to the relative excess of ATPase[2] compared with hexokinase, and the strong inhibition of the former enzyme by the higher alcohols and toluene, by azide, *etc.*, are the same in all types of tumor. In consequence, the effect of these narcotics and poisons in eliciting a steady and high rate of glycolysis is also the same. However, some regular quantitative differences between sarcoma and carcinoma were found in the rates of glucose and fructose metabolism.

Besides the enzyme systems tested in the preceding paper the activity of aldolase was likewise measured in various types of tumor. The low content of aldolase in tumor tissue compared with muscle was first demonstrated by Meyerhof and Lohmann in 1934 (1) in a survey of

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[1] This work was supported by grants from the American Cancer Society (recommended by the Committee on Growth); the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service; and the David, Josephine and Winfield Baird Foundation.

[2] Abbreviations used: ATP = adenosinetriphosphate; ATPase = enzyme splitting the first labile group of ATP; pyro P = difference between 7 min P and direct P; HDP = hexosediphosphate; HMP = hexosemonophosphate; DPN = diphosphopyridinenucleotide or pure cozymase.

the approximate content of zymohexase (= aldolase + isomerase) in various mammalian organs. According to a rough estimate, mouse carcinoma contained only about 2.0% as much zymohexase as the same weight of rabbit muscle. Warburg and Christian, in 1943 (2), found zymohexase similarly low: in Jensen sarcoma of rat there was only 4.8% of the content of rat muscle on the basis of tissue dry weight. Investigators in this country (3,4)[3] partly confirmed and partly contradicted these findings. Although, in absolute units, the tumors which were at our disposal here contained 3-4 times as much enzyme as we formerly found, the low zymohexase content of tumor compared with muscle has been confirmed throughout the following.

METHODS

Besides the transplanted rat sarcomas No. 304 and No. 231, carcinomas of mouse strain C_3H, tumors No. 5 and No. 6; sarcoma strain No. 241 of Black agouti mouse, C57; sarcoma strain No. 1BA of Bagg albino mouse; and sarcomas No. 13 of mouse strain C_3H were used.[4]

The methods for determining the activity of hexokinase, ATPase, and total glycolysis were the same as in the foregoing paper.

Determination of Aldolase

Aldolase was measured as formerly (5,6,7). Because the triosephosphate isomerase is always present in great excess, this method, which actually measures the zymohexase complex of both enzymes, gives a measure of the amount of aldolase. The triosephosphate formed in a given time is determined by the alkali-saponifiable phosphate. Either the initial rate of the equilibrium reaction is measured, or the nearly constant rate obtained by intercepting the triosephosphate with 0.05 *M* KCN (formation of cyanhydrin). Both methods were introduced by us; the latter modification was used for measuring the total content of HDP with excess of aldolase (8, *loc. cit.*, p. 117). This modification was later used by Green *et al.* (9) for determination of purified aldolase with excess of HDP. Moreover, both of these procedures give nearly the same rate of reaction (10).[5] Finally, the reaction rate can be determined by the amount of methylglyoxal formed from triosephosphate by acid hydrolysis, as we have likewise shown (5; 11, *loc. cit.*, p. 17). However, this latter method is somewhat less accurate and need be employed only in the presence of large amounts of inorganic phosphate.

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[3] We thank Dr. Lehninger for a personal communication of his results.

[4] We are indebted to Dr. Margaret Lewis of the Wistar Institute for supplying us with these tumors.

[5] However, in the present experiments we found that 0.05 *M* KCN often inhibits somewhat, and that the measurements in the absence of KCN are more reliable.

Dounce and Thannhauser-Beyer (3), who apparently have overlooked all our reports concerned with triosephosphate, zymohexase, and aldolase, determine the methylglyoxal after acid hydrolysis and criticize the determination of alkali-labile P as nonspecific. This criticism is unwarranted. If the enzyme extract is either poisoned with iodoacetic acid or dialyzed, which is a necessary part of the method, no other alkali P can form, and no triosephosphate formed can disappear. Moreover, any neglect of these precautions affects the determination of aldolase content by means of methylglyoxal in exactly the same way as the determination by alkali-labile P.

In the present paper we determined the content of aldolase in the presence of iodoacetic acid, with and without KCN, pH 8.2. When the initial rates in the absence of KCN were higher, they were used for calculation of the activity.

I. GLYCOLYSIS IN HOMOGENATES AND EXTRACTS OF VARIOUS TUMORS

The glycolysis in homogenates from sarcoma No. 1BA of Bagg albino mouse (fresh tumor) is shown in Fig. 1. HDP and HMP both react. Glucose (not shown in the figure) gives practically the same result as the blank. Pyruvate (PY) increases the turnover rate considerably. Moreover, the rates of HMP turnover in homogenate and extract (E) are identical: $Q_{La} = 80$ for 0.5 hr. and 60 for 1 hr.

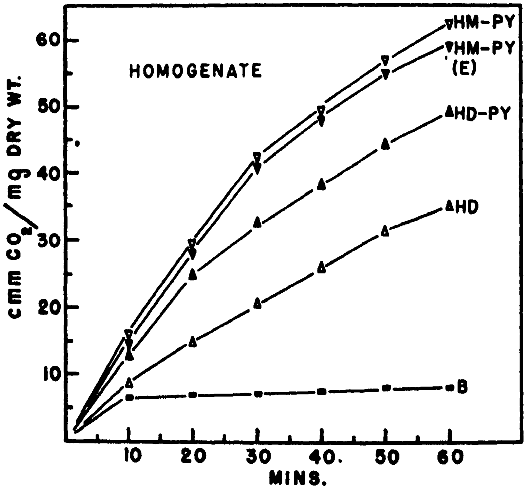

FIG. 1. Breakdown of hexosephosphates in homogenates and extracts of sarcoma of Bagg albino mouse (Exp. No. 339). The same incubation mixture as in the other experiments; 1.3 cc. total volume; 60 γ pyro P of ATP and 400 γ DPN tipped in. B = blank. HD = HDP with 1.4 mg. hexose (no pyruvate). HD-PY = the same with 0.1 cc. of 0.1 *M* pyruvate. HM-PY = Robison ester with 3 mg. hexose + pyruvate. HM-PY (E) = the same in the extract from the same homogenate.

We refrain, for the sake of brevity, from reproducing the experiments with octyl alcohol which show that the inhibition of ATPase and the eliciting of a steady glycolysis of free sugars are quite similar with all types of tumors and closely resemble those described in the foregoing paper. But the same is not exactly true of the differences in the metabolic rates of the various sugars in the presence of octyl alcohol and the influence of sugar concentrations. With one type of tumor a clear quantitative difference in the relation of fructose to glucose turnover compared with all other tumors was observed. The mouse carcinoma (C_3H) homogenate shows a turnover rate for 0.25% fructose of 80–100% of that of glucose, and high concentrations of fructose give exactly the same values as glucose. Such an experiment is shown in Fig. 2.

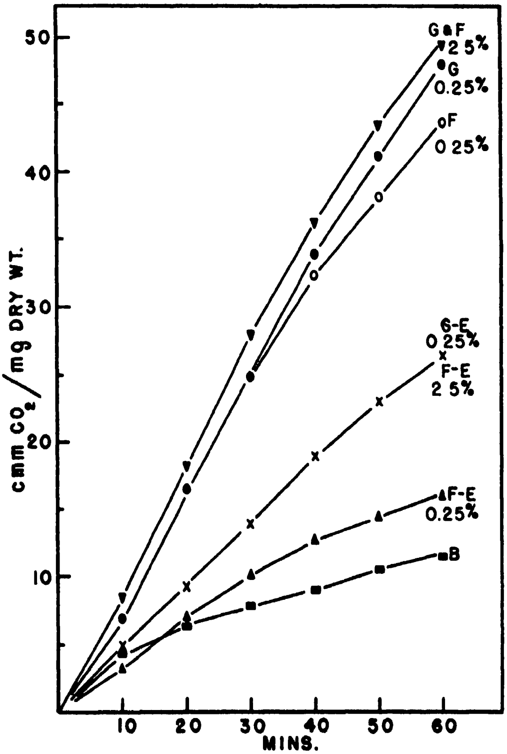

FIG. 2. Glycolysis of homogenates and extracts of mouse C_3H carcinoma strain No. 5. (Octyl alcohol) B = blank. G = glucose in homogenate. F = fructose in homogenate. G-E; and F-E = the same in centrifuged extract (Exp. No. 465).

In this case (Exp. No. 465) 0.25% fructose gives 90% of the rate of 0.25% glucose in the homogenate. The Q_{La} value of 45–50 corresponds to that obtained with glucose in homogenate of rat and mouse sarcoma under similar conditions, but with fructose all types of sarcoma would show a very low rate, corresponding to that of Fig. 7 of the foregoing paper. However, this difference is only a relative one. As the curves G-E and F-E demonstrate, 0.25% fructose reacts in centrifuged extracts of carcinoma No. 5 more slowly than 0.25% glucose, which itself is metabolized only half as fast as in the homogenate. Homogenates of the carcinoma strain No. 6 sometimes gave an even higher rate with fructose than with glucose (0.25% and 2.5%). But, even in this case, the rates were reversed and followed the normal pattern if the amount of added ATP, which, in the described experiments, had been equal to about 70 γ pyro P/cc., was lowered to 15 γ pyro P.

Some Q_{La} values obtained with homogenates of mouse carcinoma in the presence of inhibitors of ATPase are collected in Table I.

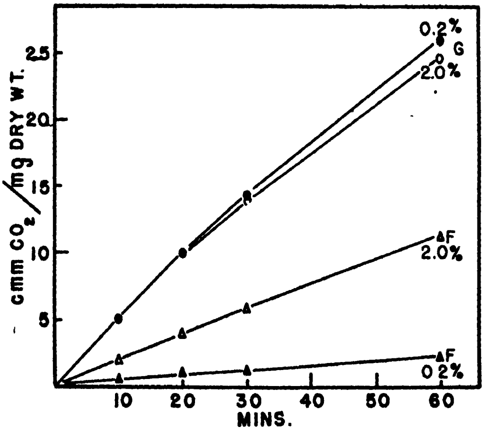

Fig. 3. Glycolysis in slices of carcinoma No. 6. G = glucose. F = fructose (Exp. No. 480).

Glycolysis in tissue slices of these mouse carcinomas does not differ from that of sarcomas. This is shown in Fig. 3. Two-tenths per cent fructose is metabolized at only 10% of the speed of glucose. If we apply here the same reasoning that we did for the analogous case of brain extract (12), we must assume that the acting concentration of the ATP in the living cell is very low.

II. ACTIVITY OF HEXOKINASE

The facts described in the foregoing are also borne out by the hexokinase activity measured in the presence of NaF (Table II). If the sugar concentration is high, transphosphorylation to fructose and glucose in carcinoma extract is equal, in contrast to sarcoma extract (see 471c, 491b for carcinoma and Table I of the preceding paper for rat sarcoma).

TABLE I

Q_{La} Values of Homogenate of Mouse Carcinoma (C_3H) with Glucose and Fructose
0.3 cc. homogenate in 1.5 cc. total volume. (100 γ pyro P of ATP.)

No.	Tumor strain	Sugar	mg	ATPase inhibitor used	Q_{La} for 30′	Q_{La} for 60′
447	Ca No. 5	—	—	sat'd. octyl alcohol		12.8
		Glucose	4	sat'd. octyl alcohol	49.5	44.5
		Fructose	4	sat'd. octyl alcohol	44.5	37.0
		Fructose	40	sat'd. octyl alcohol	57.2	48.5
465	Ca No. 5	—	—	sat'd. octyl alcohol		11.7
		Glucose	4	sat'd. octyl alcohol	51.2	48.0
		Glucose	40	sat'd. octyl alcohol	55.5	49.5
		Fructose	4	sat'd. octyl alcohol	50.5	43.5
		Fructose	40	sat'd. octyl alcohol	56.0	49.5
497	Ca No. 6	—	—	sat'd. decyl alcohol		9.8
		Glucose	3	sat'd. decyl alcohol	54.0	53.0
		Glucose	30	sat'd. decyl alcohol	63.4	56.2
		Fructose	3	sat'd. decyl alcohol	47.8	47.8
		Fructose	30	sat'd. decyl alcohol	70.5	56.5
497a	Ca No. 6	Glucose	3	0.015 *M* azide	60.5	64.0
		Fructose	3	0.015 *M* azide	64.0	57.6

If the sugar concentration is as low as 0.05%, transphosphorylation to fructose in sarcoma extract is extremely weak, especially if the disappearance of free sugar is measured directly (see Table II, no. 462), but with carcinoma extract the reaction of 0.05% fructose is still at least 50% of that of glucose.

Again, as was shown with rat sarcoma, the activity of hexokinase is mirrored in the activity of ATPase as measured by the splitting off of inorganic phosphate. In those cases in which transphosphorylation to

TABLE II

Activity of Hexokinase in Various Tumors

(NaF = 8×10^{-3} *M*) Total volume = 1.1 cc.

No.	Tumor strain	Inhibitor of ATPase	Added ATP pyro P	Added		Time	Sugar consumed	Decrease 7' P	Increase inorg. P
				Sugar	mg.				
			γ			min.	γ	γ	γ
462	Rat sarcoma	Octyl alcohol	126	—	—	12		− 7	+36.1
				Glucose	0.5	12	−213	−46.7	+16.0
				Fructose	0.5	12	− 5	−14.0	+29.3
471									
a	Mouse carcinoma No. 5	Octyl alcohol	135	—		10		6.8	+80.0
				Glucose	0.5	10	−121	−25.4	+41.5
				Fructose	0.5	10	− 56	−14.0	+54.2
b	Mouse carcinoma No. 5	—	135	Glucose	0.5	10	−146		
				Fructose	0.5	10	− 89		
c	Mouse carcinoma No. 5	Octyl alcohol	135	Glucose	4.0	10		−24	+45.3
				Fructose	4.0	10		−24	+37.6
495									
a	Mouse carcinoma No. 6	Decyl alcohol	134	—	—	15		− 1	+67.9
				Glucose	0.5	15		−37.2	+35.5
				Fructose	0.5	15		−24.0	+39.2
b	Mouse carcinoma No. 6	Decyl alcohol	134	Glucose	4.0	15		−33.8	+36.2
				Fructose	4.0	15		−39.5	+31.2

fructose is much less than to glucose, fructose inhibits the liberation of inorganic phosphate very little compared with the blank, but glucose does so strongly (No. 462). Where transphosphorylation to both sugars is nearly equal (Nos. 471c and 495), phosphate splitting is equally inhibited by both sugars.

While other intermediary enzymes of the glycolytic cycle were not systematically investigated, some differences in stability among the homogenates of various types of tumors were observed indirectly, especially with respect to the influence of pyruvate. As was discussed in the foregoing paper, pyruvate has a favorable effect on lactic acid formation if any of the enzymes responsible for transformation of phosphoglyceric acid to pyruvic acid become relatively inefficient. Pyruvate increases the glycolytic rate greatly in homogenates of rat sarcoma and, to a lesser degree, in mouse sarcoma; in the strain No. 241 of Black agouti mouse C57, the influence is especially small. In Fig. 4 some curves are reproduced from homogenates of this tumor which had been frozen for 2 days.

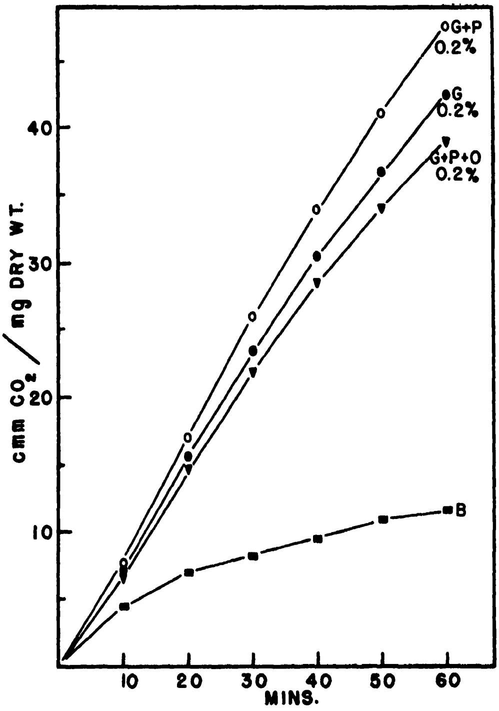

FIG. 4. Glycolysis in sarcoma No. 241 of Black agouti C57. Sarcoma frozen 2 days. B = blank. G = glucose without pyruvate. G + P = glucose with pyruvate (8×10^{-3} *M*). G + P + O = glucose with pyruvate and saturated octyl alcohol (Exp. No. 467).

In this case, as in Fig. 3 (curve E*) of the foregoing paper, the rate of glycolysis of glucose is constant for an hour in the absence of pyruvate (curve G, Q_{La} = 42.5). Nearly as much lactic acid forms as in the presence of pyruvate (curve G + P, Q_{La} = 47). Moreover, the metabolic rates are even higher than in the presence of octyl alcohol (G + P + O), while with 0.007 *M* azide the curve, which is not shown in Fig. 3, coincides with G + P. Because this is more a question of stability of the enzymes than of their activity in the fresh tissue, such variations appear of secondary importance.

III. ACTIVITY OF ALDOLASE

Since 1934 a great many papers have been published from the Heidelberg Institute (1934–1938), from the Institut de Biologie Physico-Chimique in Paris (1938–1940), and from this department (1942 to date), concerning the 2 partial enzymes of the zymohexase complex, aldolase and isomerase. The results need not be reviewed here. It has also been shown at a very early date (1) that skeletal muscle is by far the richest source of the enzymes compared with all other organs and cells, and that the next richest source is yeast. Muscle extract has, therefore, been used by all authors as source material for the purification of aldolase as well as of isomerase (9,10,13,14).

We were prompted to measure the aldolase content of our tumors, firstly, because our former value was only a rough estimate, and secondly, to check the assertion of Warburg and Christian that aldolase might be the factor limiting the speed of glycolysis in the living tissue (2, *loc. cit.*, p. 406). Our present experiments show that this contention seems improbable. If the value for turnover by aldolase is expressed as Q_P (H_3PO_4 turnover in mm.^{3}/mg./hr. at 38°C., 31 γ P = 22.4 mm.^{3} CO_2), then the Q_P value for tumor homogenates and extracts lies between 110 and 180. For the same tumors, the Q_{La} values for tissue slices are 25–35 and for the homogenate under optimal conditions, 60–70. The excess of the rate of glycolysis in the living tissue is, therefore, 4:1, and even, in the homogenate, 2:1. This fact is important because only with an excess of the intermediary glycolytic enzymes can the speed of glycolysis be regulated by the balanced activity of the phosphorylating and dephosphorylating enzymes.

From the rates in Fig. 1 and Table I of the preceding paper a Q_P of hexokinase of about 20 and Q_P of uninhibited ATPase of about 160 would be calculated for tumor homogenate in the absence of the oxidation-reduction system. However, in the case that the products of the reaction are removed by the following steps, the reaction rates are apparently much higher. Because the Q_{La} values in tumor homogenates under optimal conditions may be 60, Q_P of hexokinase must also be 60, because the phosphorylation of glucose is a necessary step in the total reaction.

While our measurements of glycolysis and aldolase activity are strictly comparable, being made with tumor extracts, prepared in an identical way and measured at the same temperature, the comparison with older values is not so easy. The approximate determination in (1) surely gave too low a value, possibly because of the age or necrosis of the tumor. The measurements of Warburg and Christian (2) were made at 20°C. by coupling with the reduction of cozymase and determination of formation of $DPNH_2$. This procedure may entail too low values for several reasons (see 4). Because the authors assume a water content of the Jensen sarcoma of 75%, while our tumors contain 85% water, their calculated Q_P (aldolase) of 58 is only $\frac{1}{2}$–$\frac{1}{3}$ of what we find in the present experiments.

We formerly defined a unit of activity (10) for the purification of enzymes of the phosphorylating cycle as the turnover rate of 1 mg. organic P/min. at 38°C. Expressed in this way, 1 g. of wet weight of rat or mouse tumor contains 0.45-0.7 units of zymohexase. According to Warburg and Christian, their tumor would contain 0.34 units/g. wet weight.

TABLE III

Zymohexase Activity of Muscle and Tumor in the Presence of Iodoacetate
(38°C.) total volume incubated = 2.0 cc.

No.	Tissue	Final dilution of fresh tissue	Time	KCN added	Alk. P found	Enzyme unit/g. fresh tissue	Dry weight of frozen tissue	Q_P aldolase
			min.		γ		*per cent*	
A5	Rat muscle	360	3	—	55	6.55	20	1530
			6	+	101	6.2		
A3	Rabbit muscle	225	2	–	70.5	7.9	20	1720
527	Rabbit muscle	115	3	–	160	6.1	20	1330
490	Rat sarcoma No. 231	16.5	3	+	242	0.67	17	174
507	Rat sarcoma No. 231	44	2	–	61.8	0.70	17	182
494	Mouse carcinoma No. 5	26.4	3	+	167	0.735	17	182
		26.4	6	+	302	0.665	17	
503	Black agouti mouse sarcoma	51.5	2	–	39.6	0.51	17	122
		51.5	4	–	70.5	0.452	17	
		51.5	2	+	35.8	0.46	17	
507	Black agouti mouse sarcoma	52	2	–	34.2	0.44	17	116
		52	4	–	69.5	0.455	17	

The rat and rabbit muscle (water content 80%) gives, in our present experiments, a Q_P at 38°C. of 1620, corresponding 7.5 units/g. wet weight. This figure is between that of Warburg and Christian (5.6 units) and that assumed in (1). This may be partly due to the content of fat and connective tissue, which lowers the Q_P.

Table III summarizes our present measurements.[6] It may be added, that we sometimes observed a lower content of zymohexase in the same strain of tumors, but in these cases the tumor was either partially necrotic or was frozen for a day or more. The sarcoma of Black agouti mouse C57 gave consistently a lower Q_P than the other tumors.

DISCUSSION

Of all the differences encountered in the glycolysis of homogenates of various tumors, only that concerning the relative rates of glucose and fructose metabolism seems to be determined by a difference in the content of enzymes. If tumor contains two different hexokinases, glucokinase and fructokinase (15), then carcinoma would probably contain relatively more fructokinase than sarcoma. The fact that, in tissue slices there is no difference between the various types of tumor, as regards fructose metabolism, is no contradiction to this. Because we must assume that the active concentration of ATP is very small in the living cell, the affinity for fructose would be very low despite a higher content in fructokinase. It may be recalled that Boyland and Boyland (16) found, for an extract of Crocker mouse sarcoma, that 1% of fructose gave more lactic acid than 1% glucose. Their experimental conditions were very different from ours and they added an amount of adenylic acid corresponding to twice the amount of ATP in our normal set up. A comparison with our results, therefore, seems difficult.

The determination of the zymohexase content of our tumors was necessary to rule out the possibility that the aldolase was the limiting

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[6] Only representative examples are given in Table III. Altogether, we have obtained on the average, the following activities by 1 g. wet weight of tissues:

In 6 samples of muscle (rat and rabbit)...	7.5 (8.8 – 6.1) units of aldolase
In 4 samples of rat sarcoma................	0.58 units of aldolase
In 2 samples of mouse carcinoma.	0.58 units of aldolase
In 3 samples of sarcoma of Black agouti mouse.	0.40 units of aldolase
In 2 samples of rat brain	0.34 units of aldolase

The muscle tissue must be carefully homogenized. Otherwise, too low values are obtained by retention of aldolase in the tissue.

factor of the speed of glycolysis. For an exact evaluation of the situation one must bear in mind that only the 3-glyceraldehyde phosphate, and not the total triosephosphate, reacts with DPN. However, because there are about 50–100 times as many units of isomerase present as aldolase/g. of muscle (10), the speed of glycolysis would be limited by the aldolase and the formation of the total triosephosphate. The second supposition, which is inherent in any such comparison of the global Q_{La} with the Q values of the individual intermediary steps, is the assumption that the concentrations of the intermediate reactants in the steady state of glycolysis are high enough to saturate the enzymes in question and to allow their maximal activity. This assumption is, indeed, hypothetical, and probably not quite true in most instances, but without it all such considerations would be meaningless. The other possibility that the enzymatic reaction rate in the steady state of glycolysis may be higher than that measured for the isolated single steps has already been discussed in the foregoing (p. 10).

SUMMARY

1. The same peculiarities of glycolysis are observed with homogenates and extracts of various types of transplanted malignant tumor, rat and mouse sarcoma and carcinoma. The excess of ATPase over hexokinase, especially, is similar. Accordingly, a steady glycolysis of free sugar in the absence of phosphate donors is possible only with inhibition of ATPase. Higher alcohols, like octyl and decyl alcohols, and 0.01 *M* sodium azide inhibit the ATPase in all types of tumor and enable the homogenates to glycolyze at a high and steady rate.

2. The main difference between the various tumors concerns the relative rates of glycolysis of fructose compared with glucose. Extracts and homogenates of mouse strain C_3H carcinoma give nearly the same rates for 0.2% glucose and 0.2% fructose, while, with homogenates and extracts of other tumors, the rate for 0.2% fructose is only a small fraction of that of 0.2% glucose.

3. The aldolase content of the various tumors was found to be somewhat higher than in our previous estimation. However, the fact described earlier, and stressed recently by Warburg and Christian, that the aldolase content of tumor is very much smaller than that of skeletal muscle, was confirmed. Q_P aldolase in tumor is 110–180; in muscle, about 1600 (at 38°C.).

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Some Aspects of the Action of Sulfonamides. I. Binding of S^{35} Labeled Sulfanilamide by *Escherichia coli*

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INTRODUCTION

Since the discovery that *p*-aminobenzoic acid antagonizes the bacteriostatic action of the sulfonamides (1), it has been evident that the action of these drugs must involve some type of competition with the former compound. It is assumed generally that this competition is for a site on an enzyme molecule essential in the metabolic processes of the microorganism. Implicit in this picture is the postulate that complexes may be formed between the bacterial enzymes and the drug.

It has been possible to calculate *relative* affinity constants for such complexes with the sulfonamides from kinetic data (2,3), but absolute binding data are scanty. Feinstone, Williams and Florestano (4) have reported small uptakes of sulfanilamide by streptococci, whereas Havinga *et al.* (5) have been unable to detect any binding by *E. coli*. On the other hand, the inability to produce substantial inhibitions of growth of *E. coli* when large inocula are used (6) would indicate, at first glance, that a significant portion of the drug is removed from the medium by the bacteria.

In view of these conflicting indications it has seemed appropriate to investigate the problem of uptake of sulfanilamide by bacteria by a more sensitive method, one involving the use of sulfanilamide labeled with radioactive S^{35}.

EXPERIMENTAL

Synthesis of Labeled Sulfanilamide

The synthesis of many of the sulfonamides can be achieved by reacting N-acetylsulfanilyl chloride with the appropriate amine, followed by hydrolysis of the N-acetyl group. Hence, by labeling N-acetylsulfanilyl chloride, many of the sulfonamides can be prepared for tracer work. The usual method of preparing this compound, by reacting acetanilide with a 5 mole excess of chlorosulfonic acid (7) was discarded, however, since the yield on sulfur is very low, and the preparation and handling of small amounts of chlorosulfonic acid presents some difficulties. Hence the sulfonation of acetanilide was carried out according to the method of Söll and Stutzer (8). The following procedure works very well on a 14 mM scale. Since most of the steps are carried out in a centrifuge cone, only 2 transfers are involved.

Preparation of Labeled Sulfuric Acid

In a 100 ml., 3-necked flask, fitted with a dropping funnel, a gas inlet tube and a reflux condenser, was placed 7 mM of FeS and 1 millicurie of S^{35} as Na_2S.[1] The condenser was fitted with 2 bubblers in each of which was placed 5 ml. of conc. HNO_3. A slow stream of N_2 was passed through the system, and 20 ml. 2.5 M HCl was slowly added through the dropping funnel to release H_2S. Finally, the solution was boiled for ½ hr. The H_2S which was swept into the bubblers was thus oxidized to a mixture of sulfate and sulfur.

When all of the H_2S had been generated, the contents of the bubblers were transferred to a 125 cc. Erlenmeyer flask containing 7 mM of finely divided sulfur, with the aid of two 5 ml. portions of conc. HCl. The aqua regia mixture oxidized the sulfur to sulfate.

The H_2SO_4 was recovered by adding the acid mixtures, a drop at a time, to a 50 ml. centrifuge cone suspended in a stirred oil bath maintained at 160–170°C. The Erlenmeyer flask was washed out thoroughly with portions of HCl and water. Heating of the concentrated H_2SO_4 was continued for 15 min. after boiling had ceased, to insure removal of traces of nitric acid.

N-Acetylsulfanilic Acid

The H_2SO_4, obtained as described, was chilled to 0°C. Five ml. of ice cold acetic anhydride and then 2 g. of acetanilide were added to the cold acid. The mixture was heated on a water bath to 80–90°C. A precipitate of N-acetylsulfanilic acid appeared in 15–20 min. and was isolated by chilling, centrifuging, and warming the separated solid at 70°C. *in vacuo.*

N-Acetylsulfanilyl Chloride

To the sulfonic acid, suspended in 15 ml. of dry chloroform, 8 g. of PCl_5 was added in small portions. After the vigorous reaction subsided, the mixture was heated to 45°C. until the evolution of HCl ceased. The resulting solution was cooled and treated with ice-water to decompose the $POCl_3$ and PCl_5. The water layer was then siphoned off. Chilling the chloroform solution usually induced crystallization of the N-acetylsulfanilyl chloride. Occasionally, however it was necessary to add a few drops of petroleum ether to the chloroform. In either case, the product obtained was centrifuged and the solvent decanted.

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[1] The active Na_2S was obtained from the Clinton Laboratories of the Atomic Energy Commission. It had a specific activity of 0.38 millicurie/mg. sulfur.

Sulfanilamide

The precipitate of N-acetylsulfanilyl chloride was mixed carefully with 10 ml. of 7 *M* NH_4OH and heated to boiling. The resultant mixture was chilled to 0°C. and centrifuged to separate the N-acetylsulfanilamide. Excess ammonia was removed by decanting the supernatant liquid, and by subjecting the residue to vacuum. Removal of the acetyl group was achieved by hydrolyis in 5 ml. of 2.5 *M* HCl until 5 min. beyond the time the solution became clear. The solution was then treated with 200 mg. of Norit A, filtered, and the charcoal washed with two 1 ml. portions of 2.5 *M* HCl. When the filtrate was brought to neutrality with 6 *M* NaOH, sulfanilamide precipitated and was filtered out. The crude product was recrystallized from 95% ethyl alcohol. M. p. 161–162°C. (corrected). Yield: 0.75 g. recrystallized sulfanilamide, 31% based on initial sulfur.

Analyses:[2] Calcd. for $C_6H_8O_2N_2S$: C, 41.8; N, 16.3. Found: C,42.0; N, 16.3.

BINDING STUDIES

The uptake of labled sulfanilanide by *E. coli* was examined in two different ways.

(A). Fifty ml. portions of a suspension of *E. coli* containing 0.9 billion bacteria/ml. were incubated for 6 hr. with 150 mg. of labeled sulfanilamide. At the end of this time the bacteria were centrifuged down, washed twice with 10 ml. distilled water on the centrifuge as rapidly as possible, and the entire residue used for a determination of radioactivity as described previously (6). No activity was detected, regardless of whether the bacteria were suspended in nutrient medium or in saline during the incubation. Similar results were obtained with only a single washing.

(B). In the second type of experiment the bacteria were incubated with the active sulfanilamide as described under (A). Bacterial suspensions of over two billion cells/ml were used, with a sulfanilamide concentration of 184 mg.-%. After incubating at 37°C. for 3 hr., the suspensions were allowed to stand at room temperature for 1 hr. Then 1 ml. aliquots were pipetted and analyzed for total radioactivity. The remaining suspensions were then centrifuged, and aliquots of the clear supernatants were taken for analysis. All the analyses were performed in triplicate. The results are assembled in Table I. No significant removal of sulfanilamide from the medium is observed.

TABLE I

Lack of Uptake of Labeled Sulfanilamide by E. coli

Exp. no.	Total activity[a]	Activity in supernatant
1	9.9	9.8
	9.1	10.1
	10.4	
	Av. 9.8±0.5	Av. 10.0±0.2
2	9.2	9.7
	10.4	8.9
	8.8	8.6
	Av. 9.5±0.6	Av. 9.1±0.4

[a] The activity is expressed in terms of (divisions/second) × 10^3 as measured by a Lauritzen electroscope.

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[2] Microanalyses were performed by Miss P. Craig.

DISCUSSION

As mentioned above, no radioactivity could be detected in the *E. coli* by the direct procedure, (A). Since the 2 washings were carried out very rapidly, it is unlikely that the drug could have diffused out of the cells.

In procedure (A) the total activity of added sulfanilamide was of the order of 2500 times the background rate of discharge of the electroscope. Since samples of 5 times the background can be determined with good accuracy (9) with this instrument, we could have determined 0.2% of the added sulfanilamide and could have detected an even smaller fraction. Hence, we must conclude that less than 0.2% of the sulfanilamide is bound by the bacterial cells. It should be pointed out, however, that this sensitivity would allow 3×10^6 molecules to be attached to each microorganism and still escape detection.

Since it seemed possible that the sulfanilamide might be bound only to the surface of the bacteria, and hence might be lost, even with very rapid washing, the second type of experiment was carried out. Clearly, if any substantial quantity of sulfanilamide is bound to the cell surface, the samples taken after centrifugation should show less radioactivity than those containing the bacterial suspension. Here again, however, no binding was detected. It is essential to point out, however, that the sensitivity of this second type of experiment is far less than that of (A). Since successive determinations of activities (Table I) show average deviations of about 5%, the precision of the radioactive analyses is of the same order of magnitude. It is thus apparent that binding of this fraction of the added sulfanilamide could escape detection by method (B).

Nevertheless, within the limits outlined, the quantity of sulfanilamide bound is too small to be detected. It has seemed necessary, therefore, to make a careful examination of the dependence of degree of bacteriostasis on the size of the initial inoculum. The results and implications of such growth studies and related experiments are described in the next paper.

ACKNOWLEDGMENT

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SUMMARY

A synthesis of sulfanilamide labeled with S^{35} is described which gives a yield of 31% pure product starting with 14 m*M* of Na_2S. This method is readily adaptable to the synthesis of any sulfonamide which can be prepared from N-acetylsulfanilyl chloride.

The binding of sulfanilamide by *Escherichia coli* was studied with this labeled sample. It is concluded that less than 0.2% of the sulfanilamide can be within the cells, and that less than 5% can be attached to the cell surface.

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Some Aspects of the Action of Sulfonamides. II. Rate-Controlling Processes in Growth Studies with *Escherichia coli*

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INTRODUCTION

In the preceding paper (1) it has been shown that the quantity of sulfanilamide removed from the medium by *E. coli* is immeasurably small. On this basis, one would not expect the degree of inhibition of growth by sulfanilamide to depend on the size of the initial inoculum; for if the formation of the enzyme-drug complex does not remove a significant portion of the free sulfonamide, the ratio of free enzyme to enzyme-drug complex should be the same, no matter how much enzyme is present, *i.e.*, no matter how many microorganisms are introduced. Consequently, the ratio of inhibited to growing cells should be independent of the size of the inoculum.

Since earlier work (2) indicated that high concentrations of sulfanilamide failed to produce marked inhibition of growth when massive inocula were used, an inconsistency with the binding experiments became evident. It has seemed appropriate, therefore, to make a more detailed investigation of the dependence of inhibition on the size of the inoculum. The results of these experiments have suggested others, in turn, which have led to a clarification of the rate-controlling process in these growth-inhibition phenomena.

EXPERIMENTAL

All of the data cited here have been obtained with a colony of *Escherichia coli* which came originally from the Northwestern University Medical School. The bacteria were grown overnight on Bacto-nutrient agar, and were then suspended in the complete nutrient medium described by MacLeod (3).

The relative concentrations of bacteria were determined by turbidity measurements with a Hellige-Diller photoelectric colorimeter.

RESULTS AND DISCUSSION

Qualitative Considerations

Growth studies have been made, at various sulfanilamide concentrations, for a series of inocula varying over a 15-fold range. The essential portion of these data can be summarized, as in Table I, as the percentage of growth, compared to the control, at the end of 6 hr. This 6 hr. interval was chosen because it was in the logarithmic period, or, in the case of the largest inoculum, just at the beginning of the stationary phase, in the control tubes containing no sulfanilamide.

TABLE I

Effect of Size of Inoculum on Sulfanilamide Inhibition of E. coli

Initial inoculum, billions/ml.	Sulfanilamide conc., mg.-%.	Growth at end of 6 hr. (control = 100)
0.05	25	26
0.28	50	61
0.28	100	57
0.25	200	48
0.30	300	37
0.50	100	70
0.50	200	60
0.50	300	49
0.73	200	78
0.75	300	69

Even a superficial glance at Table I shows that the larger inocula require higher concentrations of sulfanilamide to establish a given degree of bacteriostasis. This point is emphasized further by a comparison of the 3 experiments with 200 mg.-% sulfanilamide, for example. As the size of the inoculum is increased from 0.25 to 0.50 to 0.73 billions/ml., the percentage of growth increases from 48 to 60 to 78. Similar trends may be observed in a comparison of different inocula containing 100 and 300 mg.-% sulfanilamide, respectively. Thus, there is a decrease in the bacteriostatic power of sulfanilamide as the size of the inoculum is increased, despite the fact that the bacteria do not remove a significant quantity of the drug from the medium.

This apparent contradiction in experiments can be resolved by a more detailed consideration of the induction period for the action of sulfanilamide.

The view generally accepted to account for the delayed action of this drug assumes that sulfanilamide stops the production of an essential metabolite; but since the bacteria have a reserve supply of this metabolite, they are able to continue to grow until this substance is exhausted. The specific nature of this hypothetical metabolite has been considered by several workers (4,5,6). Although each of these groups differs in some details, implicit in each point of view is the assumption that the drug acts instantaneously in stopping the production of the metabolite.

It is recognized, of course, that instantaneous cessation of production of metabolite does not imply immediate suspension of growth. Nevertheless, the data presented in Fig. 1 are difficult to reconcile with the assumption that metabolite production is stopped immediately upon introduction of the drug.

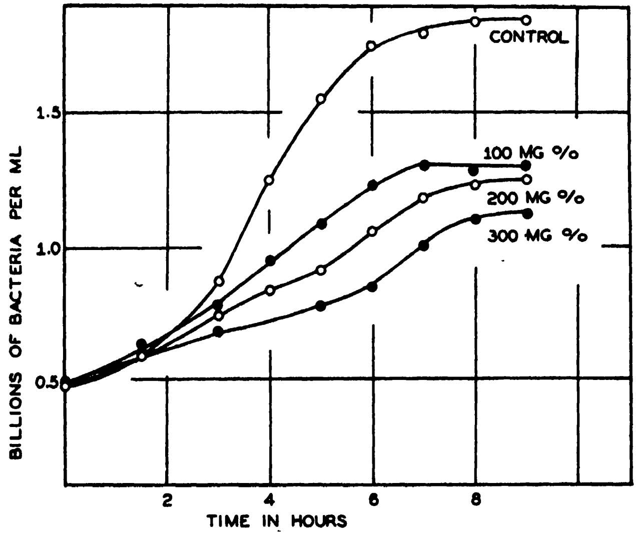

FIG. 1. Growth curves for *E. coli* in various concentrations of sulfanilamide.

The growth curves indicate that, at a constant initial inoculum, the extent of growth varies inversely with the quantity of drug present. On the other hand, as is evident from the curves in Fig. 1, there is sufficient sulfanilamide present even at the lowest concentration (100 mg.-%), to stop the growth of the microorganisms completely, after about 8 hr. From the latter observation, it follows that the inhibition of production of metabolite by sulfanilamide is complete, for if it were significantly incomplete, a complete leveling of growth should not occur at the end of the 8 hr. interval. If the action of the drug (with regard to inhibition of metabolite production) is also instantaneous, the observed growth should then be the same for each of the 3 drug concentrations, for the growth obtained must be due only to the constant content of the hypothetical metabolite present at the time the drug was introduced. Actually, striking differences in total growth are observed at the different drug levels. Such behavior seems at variance with an hypothesis of a constant initial reserve supply of metabolite in each microorganism.

It seems worthwhile, therefore, to consider the possibility that the induction period in the action of sulfanilamide may be due to a slow process in the action of the drug itself. In fact, this alternative was considered by the Dutch workers (4), but was ruled out as implausible, largely because of the belief that growth is obtained instantaneously when a culture grown in a sulfonamide-containing medium is transferred to a drug-free medium. Our experiments, in this connection, however, lead to a contrary conclusion.

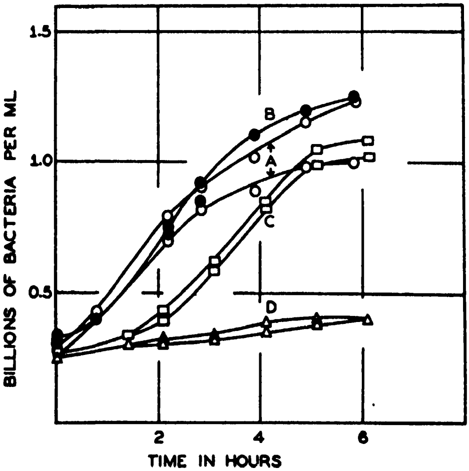

FIG. 2. Growth curves for *E. coli* after preincubation period of 6 hr. in different media. A, preincubation in medium, resuspension in medium. B, preincubation in medium, resuspension in medium containing 100 mg.-% sulfanilamide. C, preincubation in medium containing 100 mg.-% sulfanilamide, resuspension in drug-free medium. D, preincubation in medium containing 100 mg.-% sulfanilamide, resuspension in medium containing 100 mg.-% sulfanilamide.

The curves shown in Fig. 2 serve to illustrate this point. They were obtained in the following fashion. A culture of *E. coli* was incubated for 6 hr. in a complete nutrient medium with half of the tubes containing 100 mg.-% sulfanilamide. At the end of this interval, the bacteria were centrifuged down and resuspended in fresh medium, the size of the inoculum being adjusted to be about equal to that at the start of the first experiment. Curves C in Fig. 2 illustrates the growth of the bacteria which had been grown first in the 100 mg.-% sulfanilamide and resuspended in the drug-free medium. A definite lag period is evident, in contrast to the control, Curves A. Apparently a definite interval of time is required in Curves C before the effect of the sulfanilamide disappears. Thus the activity does not disappear immediately.

That 100 mg.-% sulfanilamide is adequate to maintain bacteriostasis is evident from Curves D of Fig. 2, which illustrates the very minor growth obtained when a culture grown in 100 mg.-% sulfanilamide for 6 hr. is transferred to a fresh medium which still contains 100 mg.-% drug.

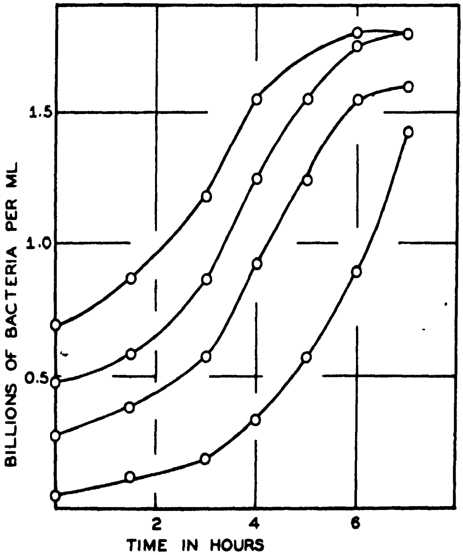

FIG. 3. Decrease in lag period with increasing size of inoculum, in growth of *E. coli*. The size of the inoculum is indicated by the point at zero time.

The transfer experiments thus are consistent with the point of view that the induction period in sulfonamide bacteriostasis is due to some slow process in the action of the drug itself. On this basis, it is also possible to see why the degree of inhibition at a given drug concentration decreases with increasing size of the initial inoculum. As is evident from Fig. 3, there is a very marked decrease in the lag period of the controls as the size of the inoculum is increased. If the action of sulfanilamide is a slow process, it is apparent that in the large inocula, with short lag period, the culture will be quite close to the stationary phase before the drug has had sufficient time to act. On the other hand, with small inocula the lag period of the bacteria is relatively long, and, hence, by the time the microorganisms have ended their lag phase, the sulfanilamide is in a position to exert its bacteriostatic action. Thus the drug can be quite effective when the lag phase of the bacteria is sufficiently long, *i.e.*, with small inocula, but relatively ineffective when the lag phase is short, as with large inocula.

From this point of view it follows also that even with a relatively small inoculum, sulfanilamide should be a relatively ineffective bacteriostatic agent if the lag period is reduced appreciably. Since it has been observed that a culture grown for 6 hr. and then transferred to a fresh medium shows practically no lag (Curve A, Fig. 2), a critical test of the assumption that sulfanilamide acts slowly would be to transfer a similar 6 hr. culture to a fresh medium containing sulfanilamide. This has been done and the results obtained are illustrated in Curve B of Fig. 2. It is apparent that no significant inhibition is obtained when the second medium contains 100 mg.-% sulfanilamide, despite the fact that this concentration of drug is quite capable of producing a very substantial inhibition when the same initial inoculum is grown under conditions where it is subject to a lag phase (Table I).

The recent observations of Youmans (7) that young, rapidly-metabolizing cells are more resistant to the action of sulfanilamide than are old cells, are also consistent with the point of view outlined in this paper. As defined by Youmans, young cells refer to those which have almost reached, or are in, the logarithmic phase of growth. Under these conditions, there is no substantial lag interval during which the slow step of the sulfonamide action can occur. Consequently, the microorganisms can grow for an appreciable period with no apparent inhibition by the drug. On the other hand old cells, that is, those early in the lag phase or ones which have completed the growth cycle, will show a significant lag interval, during which the sulfonamide may take effect. Hence, the drug will seem to be much more effective against old cells. The general behavior of young and old cells thus parallels that observed in the experiments described in this paper.

Quantitative Considerations

Since the assumption that the action of sulfanilamide is controlled by a slow rate-process seems to fit the experimental observations in a qualitative manner, it has seemed worthwhile to attempt to formulate this point of view in a quantitative fashion. For this purpose it is convenient to make the following definitions:

a = number of normal bacteria, *i.e.*, those capable of reproducing, present at any time t,
a_0 = number of bacteria in initial inoculum, *i.e.*, at $t = 0$,
b = number of inhibited bacteria, *i.e.*, those no longer capable of reproducing, at time t,
k_1 = rate constant for process of division,
k_2 = rate constant for process of inhibition.

If we picture the following 2 first-order reactions occurring simultaneously,

$$\begin{tikzcd}
 & \text{more living bacteria} \\
\text{living bacteria} \arrow[ur, "k_1"] \arrow[dr, "k_2"'] & \\
 & \text{inhibited bacteria}
\end{tikzcd}$$

then the rate of increase of living bacteria during the logarithmic phase of growth should be given by the expression:

$$\frac{da}{dt} = k_1 a - k_2 a. \tag{1}$$

This expression can be integrated readily, and the following equation obtained, on the assumption that the time t is measured from the beginning of the logarithmic phase and that $a = a_0$ at $t = 0$.

$$\ln\frac{a}{a_0} = (k_1 - k_2)t, \tag{2}$$

$$a = a_0 e^{(k_1 - k_2)t}, \tag{3}$$

Turning now to the rate of increase of inhibited bacteria we may write:

$$\frac{db}{dt} = k_2 a. \tag{4}$$

If we use Eq. (3) to substitute for a in (4) we obtain

$$\frac{db}{dt} = k_2 a_0 e^{(k_1 - k_2)t}, \tag{5}$$

which can be integrated to give the expression

$$b = \frac{k_2 a_0}{k_1 - k_2}\left[e^{(k_1 - k_2)t} - 1\right]. \tag{6}$$

In our experiments, in which turbidity measurements are used, one obtains the *total* number of bacteria at any time t. If $n = a + b$ = total number of bacteria at time t, it is apparent from Eq. (3) and (6) that

$$n = a_0 e^{(k_1-k_2)t} + \frac{k_2 a_0}{k_1-k_2}\left[e^{(k_1-k_2)t} - 1\right], \tag{7}$$

or

$$\frac{n}{a_0} = \frac{k_1}{k_1-k_2} e^{(k_1-k_2)t} - \frac{k_2}{k_1-k_2}. \tag{8}$$

Thus, we have an expression for the total number of bacteria, n, as a function of time, in the medium containing the sulfonamide. It should be emphasized that this equation is applicable only *after* the lag period, since Eq. (1), upon which it is based, is the differential expression for the logarithmic phase. This restriction may be made more evident by considering the reduction of Eq. (8) in the case where the drug concentration, and hence k_2, approaches zero. For such a situation

$$\frac{n}{a_0} = e^{k_1 t}, \tag{9}$$

i.e., one obtains the usual expression for the growth of noninhibited bacteria during the logarithmic phase.

Clearly, then, one cannot expect Eq. (8) to be applicable to the growth curves in our routine experiments, since the presence of the lag phase will obscure the pertinent data. To avoid this difficulty, we have adopted the following procedure to obtain data with which to test Eq. (8).

Tubes containing 5 ml. each of bacterial suspension were maintained at 37°C. for a period of 2.5 hr., an interval definitely greater than the lag period. To each of 2 of these tubes, 10 ml. of medium, taken from a container at 37°C., was added. To each of 2 others, 10 ml. of medium containing 45 mg. of sulfanilamide at a temperature of 37°C., was added. Thus, in the latter tubes a final concentration of 300 mg.-% of sulfanilamide was obtained. The turbidity of each tube was read immediately to obtain a value for a_0, and the growth curve was followed by turbidity readings at 0.5 hr. intervals.

The experimental observations in this series are illustrated by the points in Fig. 4. Theoretical curves have been calculated from Eq. (8), and the lines in Fig. 4 show the curve obtained for $k_1 = 0.0092$ min.$^{-1}$ and $k_2 = 0.0082$ min.$^{-1}$. The agreement between experimental and calculated values is excellent over a period of 4.5 hr. for the control and even longer for the sulfonamide-inhibited culture. Deviations begin to arise only as the stationary phase of growth is reached, a region in which, once again, one cannot expect Eq. (8) to be applicable, in view of the fact that differential expression (1), upon which it is based, assumes that the control culture is in its logarithmic stage of growth. Thus, it is evident that Eq. (8) represents the experimental observa-

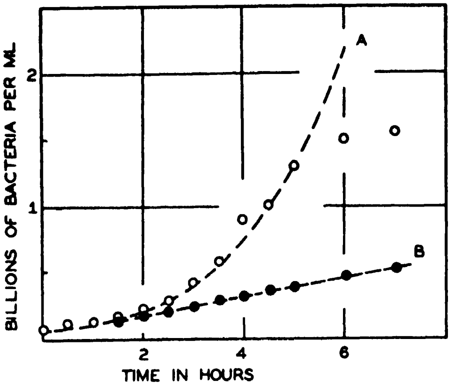

FIG. 4. Growth curves for *E. coli* after a preincubation period of 2.5 hr. to eliminate lag phase. A, in medium; B, in medium containing 300 mg.-% sulfanilamide. The circles give the observed data, the dotted lines give values calculated from Eq. (8) (curve B) and Eq. (9) (curve A) with $k_1 = 0.0092$ and $k_2 = 0.0082$ min.$^{-1}$.

tions quite adequately within the range in which the assumptions used in its derivation are applicable.

Eq. (8) is also applicable *within limits* to relatively routine experiments. As has been pointed out previously, the lag period in bacterial

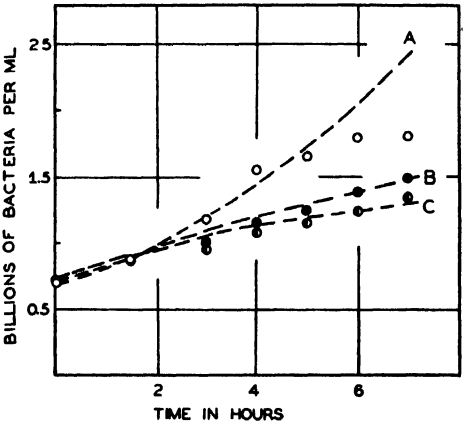

FIG. 5. Comparison of calculated and experimental growth curves for *E. coli* when large initial inoculum is used and lag period is practically eliminated. A, control; B, in presence of 200 mg.-% sulfanilamide; C, in presence of 300 mg.-% sulfanilamide. The circles give the observed data, the dotted lines give curves calculated from Eq. (8) with $k_1 = 0.003$ min^{-1}, and $k_2 = 0.004$ min^{-1} in B and 0.006 min^{-1} in C.

growth can be eliminated effectively by increasing the size of the inoculum. A series of experiments was carried out, therefore, with an inoculum in which the lag period was practically eliminated. The data obtained, with a control and with each of 2 concentrations of sulfanilamide are illustrated in Fig. 5, together with the calculated curves. The control growth can be described with a single constant, $k_1 = 0.003$ min.$^{-1}$ and the curves in the presence of sulfanilamide with k_1 and an additional inhibition constant of 0.004 min.$^{-1}$ and 0.006 min.$^{-1}$ in 200 and 300 mg.-% solutions, respectively. Thus, once again the experimental data can be fitted quite satisfactorily by an equation based on the assumption that the sulfanilamide acts slowly. It is of particular interest to note in this connection that the inhibition constant k_2 is proportional to the concentration of drug.

In view of the validity of Eq. (8) as a quantitative correlation of growth data in systems containing sulfanilamide, it may be pertinent to examine its general behavior for different possible values of k_1 and k_2. Two cases of interest may be considered. If $k_2 > k_1$, then it is evident from Eq. (8) that the first term will have a negative exponent and, hence, will approach zero with increasing time. Clearly, then, n/a_0 will approach a limiting value of $k_2/(k_2-k_1)$, *i.e.*, the growth curve will level off, even if the bacteria do not possess a stationary phase.

The second case to be considered, in which $k_2 < k_1$, shows a different behavior. From Eq. (8) it is apparent that the exponent, being positive this time, will increase without limit with increasing time, the only effect of the drug being to retard this increase somewhat at the outset, by the subtraction of the second term $k_2/(k_1-k_2)$. Thus, under these conditions, it is only the stationary phase of the bacterial growth curve which produces the plateau in reproduction. A superficial examination of an *in vitro* experiment would not distinguish between these 2 situations, for in both cases the growth in the presence of sulfanilamide would be somewhat reduced and would reach a plateau with increasing time. However, if a system could be devised, whereby the leveling off due to the stationary state could be avoided, one would predict from the above analysis that when $k_2 < k_1$ the bacteria would continue to grow at an increasing rate, even in the presence of the drug.

CONCLUSIONS

The qualitative and quantitative aspects of the experiments described both lend support to the proposal that a rate effect is involved in the action of sulfanilamide. The nature of this rate process, however, is still open to some question. Most attractive, perhaps, is the hypothesis of slow penetration of the drug through the cell wall of the microorganism. Since these experiments have been carried out with a nonionized sulfanilamide, the penetration hypothesis would indicate that even a neutral molecule may diffuse only slowly through the bacterial membrane. Without further work, however, one cannot rule out other processes within the cell as being the rate controlling steps.

Acknowledgments

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Summary

Growth curves for *E. coli* in the presence of sulfanilamide have been examined for various sizes of initial inocula. The degree of inhibition of growth by a given concentration of drug has been found to be an inverse function of the size of the inoculum. This behavior suggests that there is a slow step in the action of sulfanilamide. The presence of a slow step is indicated also by the lag in growth of bacteria transferred from a sulfanilamide-containing to a drug-free medium. Because of this slow step, the effectiveness of the sulfanilamide is decreased in experiments in which the lag phase in bacterial growth is eliminated or reduced.

A quantitative expression has been derived, based on the assumption of 2 simultaneous first-order reactions, growth and inhibition, which is capable of representing the experimental growth data in a very satisfactory fashion.

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Spectrophotometric Studies of the Oxidation of Fats. VIII. Coupled Oxidation of Carotene [1]

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INTRODUCTION

The oxidative destruction of carotene in solution is a well known phenomenon to those who work with this substance. The coupled oxidation of carotene in rancidifying oils is an economic and nutritional problem in the storage of oils and foods in which this vitamin A-active substance occurs. The oxidation of carotene, either alone in solution, in rancidifying oils, or as the result of lipoxidase action is evidenced by the loss of color. The changes in absorption spectrum accompanying the destruction of carotene in solvents have been studied in some detail by McNicholas (1), Van Der Hulst (2) and Miller (3), who showed that, as the principal absorption band of carotene decreases, absorption in the ultraviolet region increased. Hunter and Krakenberger (4) have made spectral and chromatographic studies of the oxidation of β-carotene in arachis oil and in benzene solution, and found that the products of oxidation were similar. However, no data are available on the changes in absorption spectra in relation to the oxygen uptake of unsaturated oils. It was thought that a study of the spêctral changes accompanying this oxidation might give some added information on the mechanism of the oxidation of carotene. To simplify interpretation of results, the solvent for carotene in these studies has been a pure unsaturated ester whose spectral changes accompanying oxidation are already known.

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EXPERIMENTAL

The carotene used in this study was 10% α- and 90% β-carotene from a freshly-broken ampule. Ethyl linoleate was prepared according to the method described by McCutcheon (5). All oxidations were allowed to take place under air in Warburg vessels at 37°C. Each vessel contained approximately 50 mg. of a 0.6% solution of carotene in ethyl linoleate. In the second experimental series, in which emphasis was laid on the very early stage of oxidation, approximately 260 mg. of carotene-linoleate mixture was placed in each vessel, thereby allowing more accurate measurement of oxygen uptake at the very low levels. In these experiments, samples were taken at appropriate intervals of oxygen uptake, diluted with alcohol, and examined spectrophotometrically.

RESULTS AND DISCUSSION

It was found that nearly all of the carotene was oxidized before 0.1 mole oxygen was absorbed per mole ester (Fig. 1.). In this early phase of

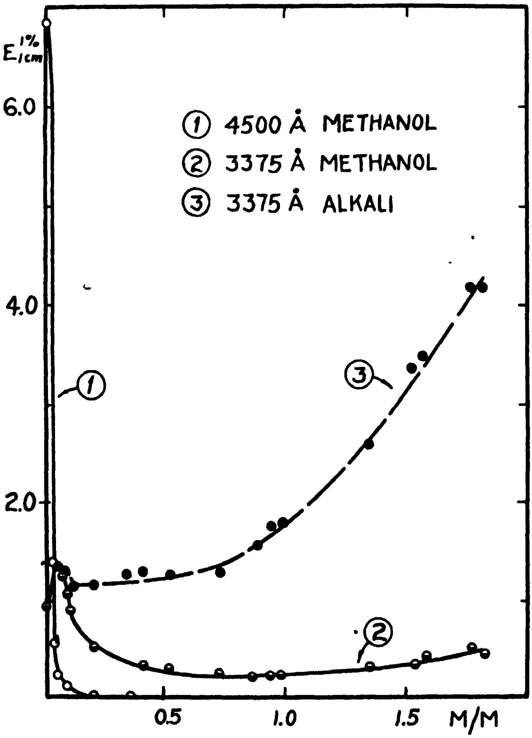

FIG. 1. Spectral changes of carotene in ethyl linoleate related to the oxygen uptake of the ester.

the oxidation, the absorption band at 3375 Å first increased and then decreased sharply, later to rise again during the oxidation process. This increase in light absorption is associated with carotene destruction and is not a result of the oxidation of linoleate itself (6). The band appearing at 3375 Å is probably the *cis*-peak of Zechmeister and Polgár (7), who have shown that isomerization of carotenoids to *cis* forms induces an absorption band in this region with a simultaneous decrease at the principal absorption band. The absorption bands at 2675 Å and 2325 Å, associated with the oxidation of linoleate, developed in the carotene-linoleate system in a manner similar to that in the oxidation of pure linoleate (6). The increase in absorption above 3000 Å upon the addition of alkali is due to a product of oxidation of linoleate.

The second series of experiments was designed to study the initial stage of the oxidation of carotene. In these experiments, the amount of carotene-linoleate mixture was increased to 250–300 mg. per vessel in order that oxygen uptake values for less than 0.02 M/M would be large enough to be measured with accuracy. The changes in absorption at 2325 Å due to the formation of conjugated linoleate peroxides, and the concurrent decrease in carotene absorption at 4500 Å are shown together in Fig. 2. It will be seen again that, before 10% of the ester was oxidized, virtually all of the carotene was destroyed.

The apparent molar extinction coefficient of the linoleate hydroperoxide formed in this experiment and calculated on an oxygen basis was 22,700. This value is to be compared with that of 18,900 for ethyl linoleate previously reported (8). It is unfortunate that carotene does not lend itself to direct measurement of its oxygen uptake because of its high melting point and its very limited solubility in solvents suitable for use in the Warburg apparatus and as solvent in spectrophotometry; for knowledge of the relationship between oxygen uptake and spectral changes in carotene oxidation would be desirable for more complete interpretation of the data presented here.

In the oxidation of linoleates, the appearance of the absorption band at 2600–2800 Å is presumably due to the presence of small amounts of unsaturated ketones. In this carotene-linoleate system, the absorption at this wavelength first showed a slight decrease, insignificant by comparison with the increase taking place in later stages of the oxidation (9). The development of the band at 2600–2800 Å is much the same whether carotene is present or not.

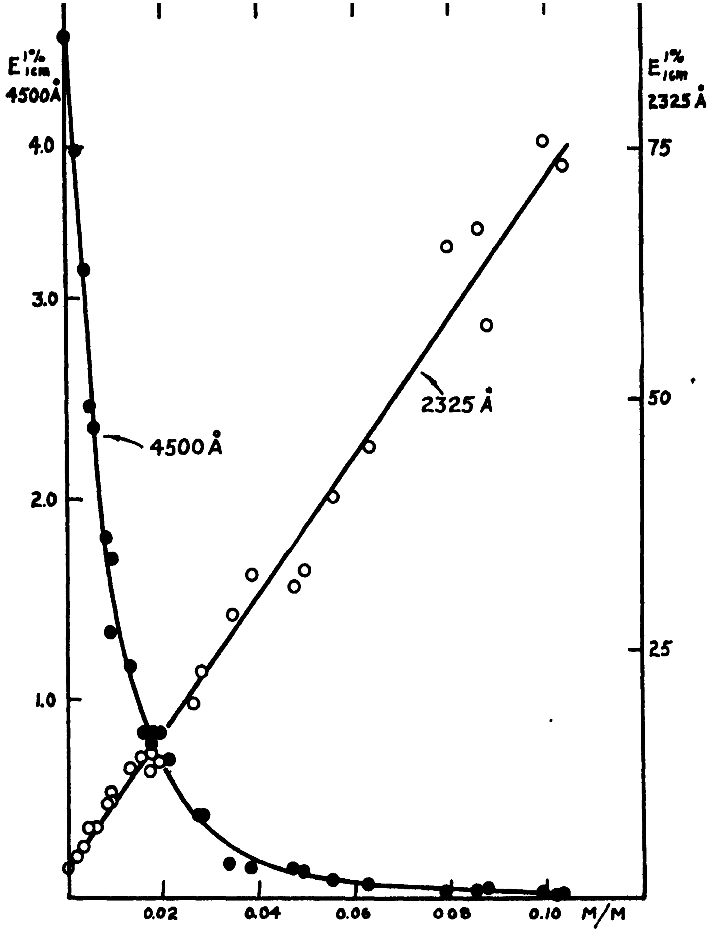

FIG. 2. Spectral changes in early stage of oxidation of carotene in ethyl linoleate related to oxygen uptake.

The initial increase in absorption at 3375 Å, which was indicated in the first experiment, is shown in Fig. 3. It is apparent that this increased absorption, although real, is transitory: The reproducibility of the values is very poor, indicating that the substance responsible for this increase is probably unstable and is affected by slight variations in experimental conditions which escaped detection. The increase is not great, but it accompanies the precipitous decrease in absorption at 4500 Å, suggesting that it may be associated with the primary product of carotene oxidation. If this band is a *cis*-peak, the oxidation of carotene must induce some *trans-cis* isomerism. This also would contribute to the decreased absorption at the major absorption band.

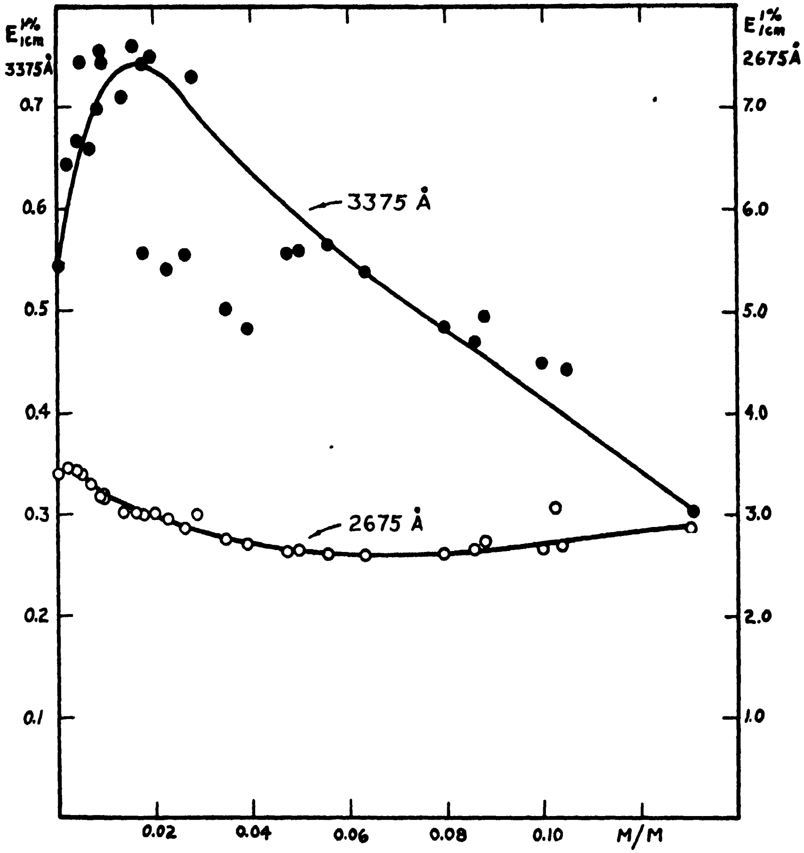

Fig. 3. Spectral changes in early stage of oxidation of carotene in ethyl linoleate related to oxygen uptake.

Preliminary experiments in which carotene, ethyl linolenate, and carotene plus ethyl linolenate were allowed to oxidize in butanol solution indicated that the chief absorption maxima of oxidized carotene lie at 2325 and 2750 Å, in the same regions where the principal absorption bands of oxidized unsaturated fatty acids lie. These results suggest that the autoxidation of carotene yields products in which only a few of the original double bonds contribute to the ultraviolet absorption. It is indeed interesting that carotene, which has 11 conjugated double bonds, should form so little detectable intermediate oxidation products with somewhat fewer double bonds in conjugation. It is as if the carotene were attacked at several points at once, reducing the long conjugated unsaturated system to an unsaturated system involving only a few conjugated double bonds. However, it is quite unlikely that such multiple attack occurs simultaneously. These results are at variance with those of Hunter and Krakenberger who found several products by chromatographic fractionation of the oxidized carotenoid. They postulated the attack at the terminal double bond of the unsaturated system to form an epoxide. It may well be that, in this present study, the oxidation is so rapid in autoxidizing linoleate that no spectrophotometrically detectable intermediates, such as those found by Hunter and Krakenberger, accumulated. In the present experiment the only spectral evidence of an intermediate having more than a few conjugated double bonds is the transitory increase in absorption at 3375 Å in the initial stage of oxidation, and this increase is probably the *cis* peak rather than a product containing 5 or 6 conjugated double bonds.

The coupled oxidation of carotene is considerably more rapid than is the oxidation of carotene in butanol solution. The virtually complete oxidative destruction of carotene in the easily-oxidizable ethyl linoleate was accomplished in about 24 hr., whereas the autoxidation of carotene in butanol required 400 hr. to reach the same stage, as judged from spectra. It thus appears that, in the medium of an oxidizing unsaturated fat, carotene oxidation is stimulated by some intermediate product of fat oxidation. From the experiments of Sumner (10), it is quite unlikely that this rapid oxidation of carotene is due to reaction of carotene and linoleate peroxide.

In the mechanism of oxidation of linoleate put forth in recent years (11,12), the existence of a free radical of linoleate is indicated. In the stabilization of this free radical, the unsaturated system becomes a conjugated one. The radical reacts with oxygen and then accepts a hydrogen atom from another linoleate to form a hydroperoxide of linoleate and another new free radical. This mechanism repeating itself is the chain reaction by which linoleate is oxidized. Any substance which furnishes a hydrogen atom and which in turn does not accept one from linoleate would stop the reaction chain. Addition of these radicals would also stop the chain oxidation. It appears that the coupled oxidation of carotene proceeds by some such mechanism. Although some evidence has been gained to suggest that carotene influences the later course of linoleate oxidation (9), it appears that, in the initial stages of the oxidation, it does not greatly affect the course of the formation of hydroperoxidolinoleate.

The spectral changes occurring in the coupled oxidation of carotene are qualitatively the same as those occurring in the autoxidation of carotene in solution (1,2,3), although the production of the various chromophores is quantitatively different. It thus appears from spectral evidence that the coupled oxidation of carotene yields the same products as does the autoxidation of carotene.

SUMMARY

Studies, in which spectral changes in carotene-ethyl linoleate mixtures were related to oxygen uptake, indicate that carotene is nearly completely destroyed very early in the oxidation, before 10% of the ester is oxidized. During the phase when carotene is being rapidly oxidized, a transitory increase in absorption at 3375 Å is observed, the only spectral evidence of a product of carotene oxidation intermediate between carotene and the final products which absorb light in the regions of 2325 and 2700 Å.

The products of autoxidation of carotene and of coupled oxidation of carotene are qualitatively similar.

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Studies on the Biochemistry of *Tetrahymena*. XII. Pyridoxine, Pyridoxal, and Pyridoxamine [1]

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INTRODUCTION

In 1945 (1), evidence was presented for the nonessentiality of pyridoxine in the nutrition of *Tetrahymena geleii.* This evidence was based on the observation that transplantable growth could be obtained in a medium which had been treated in such a way that the *Lactobacillus casei* assay for pyridoxine was negative. At that time it was necessary to add crude materials to the medium to supply the Factor II activity for *Tetrahymena.* With all of the constituents of the medium (with the exception of protogen (2,3) which is included at a concentration of 0.375 γ/ml.) composed of chemically identified substances, however, it has been possible to reexamine the B vitamin question and to check the validity of the *L. casei* assay method. This report deals with investigations of the relative activities of pyridoxine, pyridoxal (2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine), and pyridoxamine (2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine), and the changes in activities which occur during the exposure of pyridoxine to amino acids during the preparation of the medium.

EXPERIMENTAL

The organism used for this series of investigations was *Tetrahymena geleii,* strain W (3). The techniques employed have been given in detail on previous occasions (3,4). The base medium employed is given in Table I, and the terminology for the block of amino acids (SCH) is the same as that used previously (3). One item in the base medium needs some explanation. During the course of these experiments, two lots of yeast nucleic acid were used. They were identical except for age, and it is to be noted that the fresh lot possessed slight pyridoxine activity, while the older lot did not.

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[1] Aided by a grant from the U. S. Public Health Service, and a grant recommended by the Committee on Growth acting for the American Cancer Society.

TABLE I

Base Medium

(All amounts are given in γ/ml. of final medium)

L-Arginine HCl	83	Sodium acetate	1000
L-Histidine HCl	36	Ca pantothenate	0.10
DL-Isoleucine	113	Nicotinamide	0.10
L-Leucine	147	Thiamine HCl	1.00
L-Lysine HCL	116	Riboflavin	0.10
DL-Methionine	94	Pteroylglutamic acid	0.01
L-Phenylalanine	70	Biotin (free acid)	0.0005
DL-Threonine	138	Choline Cl	1.00
L-Tryptophan	28	Protogen[a]	0.375
DL-Valine	76	Yeast nucleic acid (hydrolyzed)	100
DL-Serine	157	$MgSO_4 \cdot 7H_2O$	100
L-Glutamic acid	233	K_2HPO_4	100
L-Aspartic acid	61	$CaCl_2 \cdot 2H_2O$	50
Glycine	5	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
DL-Alanine	55	$FeCl_3 \cdot 6H_2O$	1.25
L-Proline	175	$CuCl_2 \cdot 2H_2O$	5
L-Hydroxyproline	75	$MnCl_2 \cdot 4H_2O$	0.05
L-Tyrosine	67	$ZnCl_2$	0.05
L-Cysteine	3.5	Tween 85[b]	500
Dextrose	1000		

[a] Furnished by the Lederle research laboratories through the courtesy of Dr. E. L. R. Stokstad.

[b] Furnished by the Atlas Powder Company, Wilmington, Delaware.

This is explained by the fact that the nucleic acid solution is kept in a clear flask, under toluene, and no special care is taken to guard it from the ordinary light of the room while experiments are being set up. After repeated exposures to light over a number of weeks, the trace of pyridoxine activity possessed at the start completely disappears (see Figs. 1 and 2). This trace of pyridoxine activity in freshly prepared nucleic acid solutions is not detected with the *L. casei* method of assay (1).

RESULTS

In the absence of pyridoxine no growth occurs, but when graded amounts of pyridoxine HCl are added to the medium, regular increments of growth are obtained. The amount of response to suboptimal doses varies, however, depending upon the previous exposure of the pyridoxine to the amino acids of the medium. When the pyridoxine is sterilized by Seitz filtration and added aseptically to the tubes, the amount required for approximately half-maximum growth is 0.45 γ/ml. (Fig. 1). Maximum response is obtained with approximately 2.0 γ/ml. of medium. When the pyridoxine solution is autoclaved apart from the medium and then added to it aseptically, the activity is nearly doubled, for in this case the amount required is 0.25 γ/ml. for approximately

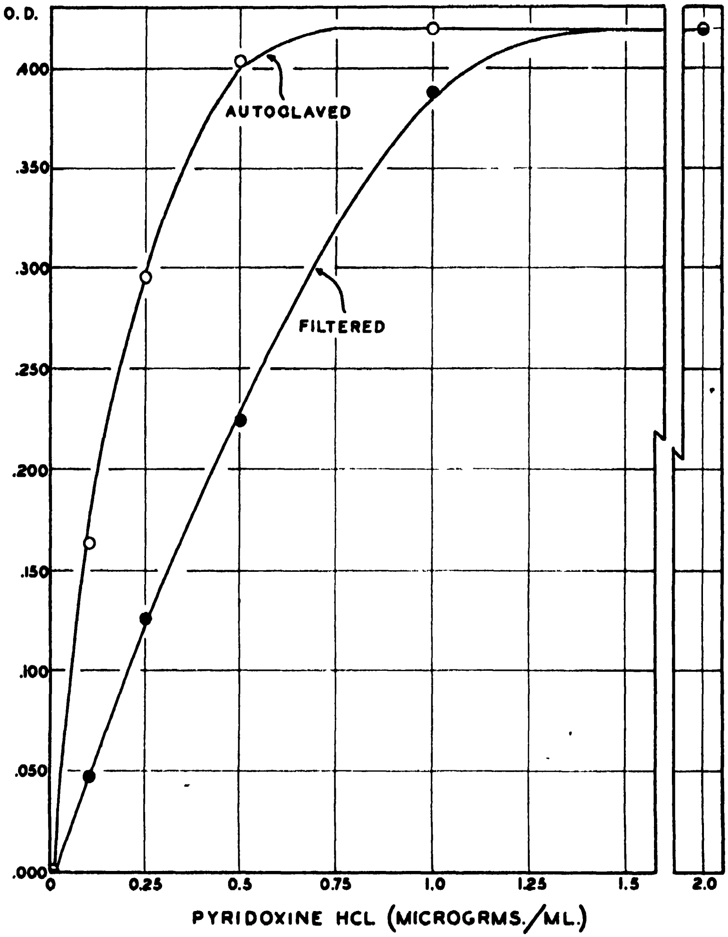

FIG. 1. Dose response to pyridoxine which has been sterilized separately by filtration, or sterilized by autoclaving with the medium for 15 min. at 15 lbs. pressure. The base medium is that given in Table I, to which has been added the essential amino acids in the following concentrations (γ/ml.): arginine 40; histidine 15; isoleucine 50; leucine 50; lysine 40; methionine 60; phenylalanine 20; threonine 50; tryptophan 16; valine 10; serine 157. Growth is recorded as optical density. Note that aged hydrolyzed yeast nucleic acid was used in these experiments (see text).

half-maximum growth (Fig. 2, "0 min."). It appears that changes (possibly to pyridoxal) take place under the influence of heat which increase its activity. Another possibility is that appreciable adsorption of pyridoxine takes place on the pad of the Seitz filter and there is no real difference in activity, simply in amount.

The activity is markedly increased when the pyridoxine is combined and sterilized with the medium. This is illustrated in Fig. 1, where the time of autoclaving was 15 min. at 15 lbs. pressure. The amount required for approximately half-maximum growth is 0.125 γ/ml., and maximum yield is obtained with 1.0 γ/ml. The activity of the pyridoxine after 15 min. of autoclaving with a medium of lower amino acid content remains the same (Fig. 2) when allowance is made for the trace of pyridoxine activity brought in with the freshly prepared nucleic acid. After 30 min. of autoclaving with the medium, the activity is increased still further, so that half-maximum growth is now obtained with 0.075 γ/ml.

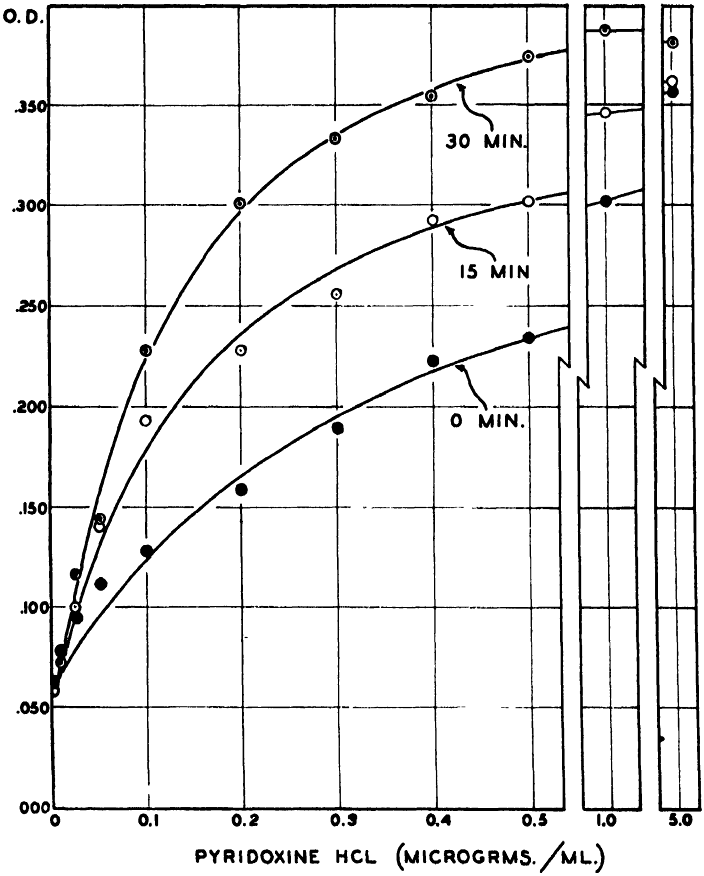

FIG. 2. Dose response to pyridoxine sterilized by autoclaving. Zero minutes = autoclaving a solution of pyridoxine and adding it to medium; 15 and 30 min. = combining the pyridoxine with the medium and sterilizing for the respective periods of time. Base medium as in Table I. Freshly prepared hydrolyzed yeast nucleic acid used (see text).

The reactions which take place under the above conditions have been discussed by Snell and his associates (5,6,7,8) and, accordingly, both pyridoxal and pyridoxamine were tested.

There appears to be no significant difference between the activities of pyridoxal and pyridoxamine when they are autoclaved with the medium, but both are well over 100 times more active than pyridoxine. This is somewhat surprising in veiw of the fact that Snell and Rannefeld (8) found that all 3 substances possessed approximately equal activity for rats. Half-maximum growth of *Tetrahymena* was obtained with the addition of 0.0007 γ/ml. of medium, and maximum yield resulted with the addition of 0.01 γ/ml. (Fig. 3). These data indicate that the animal organism, like the bacteria, utilizes the pyridoxal and/or pyridoxamine in its metabolism. Evidence has been presented that pyridoxal phosphate acts as a coenzyme in the decarboxylating

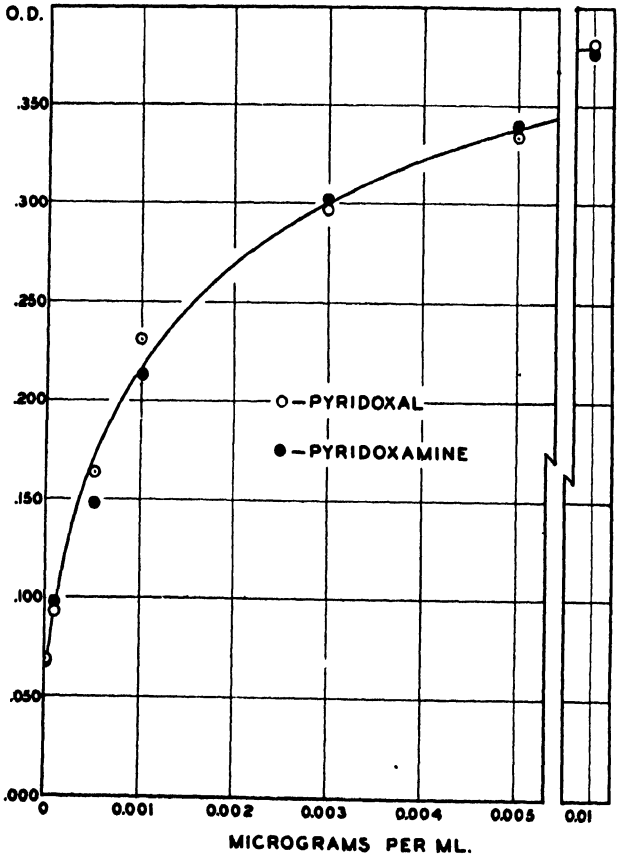

FIG. 3. Dose response to pyridoxal HCl and pyridoxamine HCl. Base medium as in Fig. 2.

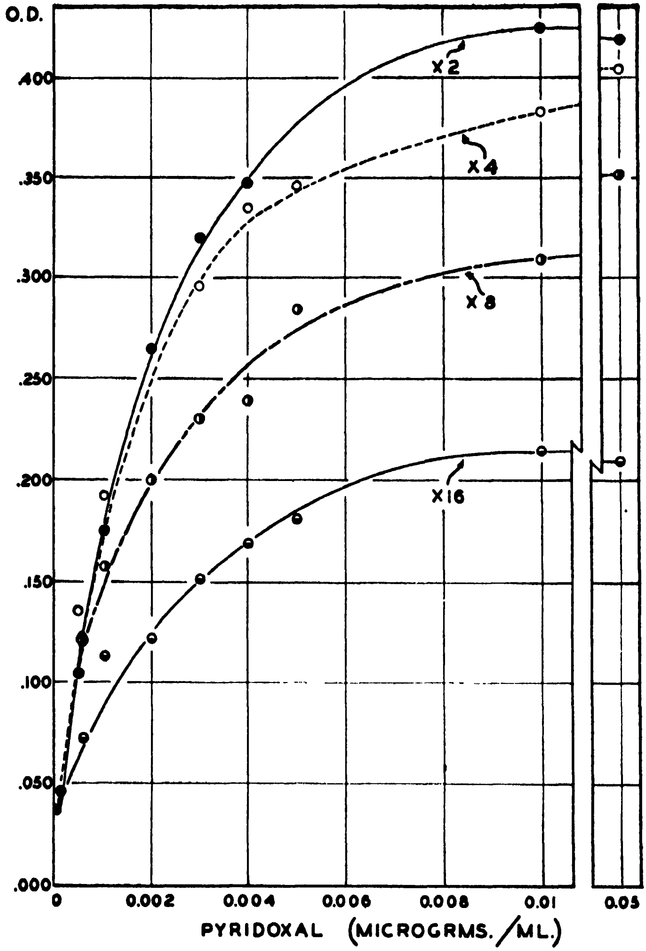

FIG. 4. Dose response to pyridoxal HCl. Base medium as in Table I, to which has been added simulated casein hydrolyzate (3) in increasing amounts.

mechanisms (9,10), and also during transamination (11,12). If this is true, then *Tetrahymena* must be able to change pyridoxine to pyridoxal, provided sufficient amounts of the former are present, and very readily accomplish the change from pyridoxamine to pyridoxal. In this connection it is interesting, and perhaps significant, to note that the activity of pyridoxamine does not change when a solution is autoclaved separately and then added aseptically to the medium, but, in the case of pyridoxal, the amount required for maximum yield is lowered to approximately 0.005 γ/ml. under like conditions. Pyridoxal, therefore, is somewhat more active when not subjected to contact with amino acids during sterilization.

The final set of experiments dealt with the dose responses to pyridoxal when tested with varying concentrations of amino acids in the base medium. To the base medium (Table I) was added simulated casein hydrolyzate (SCH) in such amounts that the total amino acid concentration was raised by approximately ⅔ (2X), 2⅔ (4X), 5⅓ (8X), and 10⅔ (16X). To each of these media, graded amounts of pyridoxal were added. While the levels of growth (Fig. 4) are influenced by the amino acid concentrations (increased at 2X and 4X and decreased at 8X and 16X, as compared with the normal base medium), the activity of the pyridoxal is approximately the same in all cases (0.0007 γ/ml. for half-maximum growth).

DISCUSSION

The results on the essential nature of pyridoxine (pyridoxal or pyridoxamine) in the nutrition of the animal microorganism, *Tetrahymena geleii*, has one very important aspect. It demonstrates the essential weakness of the *L. casei* assay method. In our previous work (1) with crude substances, precautions were taken to free any bound pyridoxine, in the materials tested, by digestion with pepsin and takadiastase (13) before all assays. In spite of this treatment enough pyridoxine remained bound, and therefore unavailable to the lactic acid bacteria, to invalidate the results. According to these tests, preparations were judged pyridoxine-free which, when added to an otherwise pyridoxineless base medium, permitted transplantable growth of *Tetrahymena*. The conclusion was therefore drawn that pyridoxine was not an essential growth factor for this organism. In reality, however, *Tetrahymena* requires large amounts of pyridoxine. These findings indicate the necessity of using lactic acid bacteria assay methods with extreme caution. Because of the battery of hydrolytic enzymes possessed by *Tetrahymena*, it may be that it can be adapted for assay procedures. Plans are formulated to explore this possibility, and, if successful, it should prove valuable for assaying for pyridoxine in foods. The basic animal nature of this microorganism makes it probable that it will respond to those substances which will prove active for animals in general.

SUMMARY

1. Quantitative investigations were carried out with *Tetrahymena geleii* to test its response to pyridoxine, pyridoxal, and pyridoxamine. Previous work had indicated that this organism did not require an exogenous source of pyridoxine, but these results were shown to be invalid.

2. When pyridoxine is sterilized by filtration and added to the medium, half-maximum growth is obtained with the addition of 0.45 γ/ml.

3. When a solution of pyridoxine is sterilized by autoclaving, and then added to the medium, half-maximum growth results with the addition of 0.25 γ/ml.

4. The activity of pyridoxine is increased when it is heated with the amino acids of the medium. Half-maximum activity was at 0.125 γ/ml. when heating was carried out for 15 min., and at 0.075 γ/ml. after 30 min. of heating.

5. Pyridoxal and pyridoxamine were found to be from 100 to 500 times more active than pyridoxine (depending upon its treatment). Half-maximum activity was at 0.0007 γ/ml.

6. Increases in the amino acid concentrations of the medium did not change the quantitative requirement for pyridoxal.

7. The inadequacy of the *L. casei* assay method for the evaluation of pyridoxine activity in crude materials is discussed.

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Studies on the Biochemistry of *Tetrahymena*.
XIII. B Vitamin Requirements [1]

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INTRODUCTION

Earlier in this series of investigations we have presented evidence for the nonessentiality of thiamine (1,2,3), riboflavin, pantothenate, nicotinic acid, pyridoxine, biotin (4), *p*-aminobenzoic acid, inositol (5), and choline (6), for the animal microorganism *Tetrahymena geleii*. Throughout this work it was necessary to add crude extracts to supply the "Factor II" needed for the growth of this organism. A reexamination of the B vitamin problem without the necessity of including crude extracts (7,8) makes it clear that our original conclusions were wrong regarding the nonessentiality of pyridoxine (8,9), riboflavin, pantothenic acid, and nicotinic acid. As has been pointed out with respect to pyridoxine (9), the earlier conclusions were based upon the negative results of the *Lactobacillus casei* assays of the treated extracts for the vitamins in question. We now know that these assay results were erroneous.

EXPERIMENTAL

Tetrahymena geleii W was used exclusively. All results are based on third serial transplants and the general techniques employed have been given elsewhere (8,9). Growth was measured turbidimetrically with a Lumetron photoelectric colorimeter (6). The base medium was the same as that given previously (9), with the addition of pyridoxine HCl (2 γ/ml.). The growth factor to be tested was omitted in each appropriate case.

RESULTS

Thiamine. Earlier work (1,2,3) established the nonessential nature of thiamine for *Tetrahymena*, provided certain conditions were met. It was shown (3) that when

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[1] Aided by a grant from the U. S. Public Health Service and a grant recommended by the Committee on Growth acting for the American Cancer Society.

D-amino acids were present in the medium, then transplantable growth would result without exogenous thiamine, but that considerable stimulation resulted upon the addition of the vitamin. Because the present base medium contained a number of amino acids as the racemic mixture, it was expected that growth would occur in the absence of thiamine. This was not found to be the case. The thiamine relationships are being further investigated.

Biotin. Repetition of the tests for the nonessentiality of biotin, using the more purified base medium, were carried out. Transplantable growth resulted in the absence of exogenous biotin, and very little stimulation occurred when it was included. Replacement of biotin by oleic acid has been established for certain bacteria (10,11) and, therefore, the tests were repeated in the absence of both biotin and Tween 85 from the base medium. Although the omission of Tween 85 reduces the yield (8), the addition of biotin had insignificant effect. When Tween, aspartic acid (12), and sodium acetate (13), were all omitted from the base medium (minus biotin) the results were not significantly different. There occurred transplantable growth and the addition of biotin at relatively high levels raised the yield only slightly (Table I).

TABLE I

Dose Response to Biotin

Amount added	Base medium	Base medium minus Tween	Base medium minus Tween, aspartic acid, and Na acetate
	Optical density after 72 hr. incubation		
γ/ml.			
0	.336	.223	.207
0.0000005	.336	.249	.211
0.0000025	.332	.233	.231
0.000005	.367	.225	.200
0.00001	.346	.235	.201
0.000025	.367	.225	.216
0.00005	.352	.229	.234
0.0001	.356	.228	.232
0.0005	.348	.244	.237
0.001	.361	.232	.262

Under the conditions of these experiments, therefore, it can be said that no requirement for exogenous biotin by *Tetrahymena* can be demonstrated, and the slight stimulation resulting from its presence is of doubtful significance.

Choline. It had been previously shown (6) that choline activity for cholineless *Neurospora* increased in a culture of *Tetrahymena*, thus indicating that the organism is capable of synthesizing choline. Tests with the present base medium demonstrate again that this organism is independent of exogenous choline.

0.0003 γ/ml. of medium (Fig. 4). This figure is based on the results of a number of separate determinations.

Use of the present medium removes the differences between the activities of the conjugated forms of folic acid, which we previously found (16). On a molar basis PGA, PTGA[2] (pteroyldiglutamylglutamic acid) and PHGA[3] (pteroylhexaglutamylglutamic acid) all have approximately the same activity. It is significant, however, that the present response to PHGA is almost identical with that found when the crude "Factor II" preparations were used.

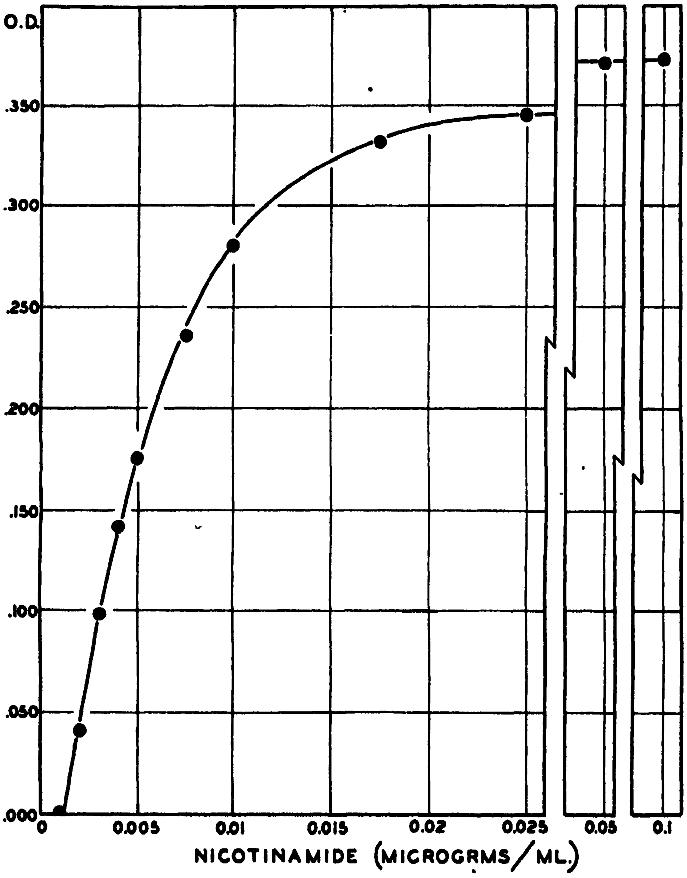

FIG. 3. Dose response to nicotinamide. Results from third serial transplant after 72 hr. incubation.

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[2] Supplied by Dr. E. L. R. Stokstad.

[3] Supplied by Dr. O. D. Bird.

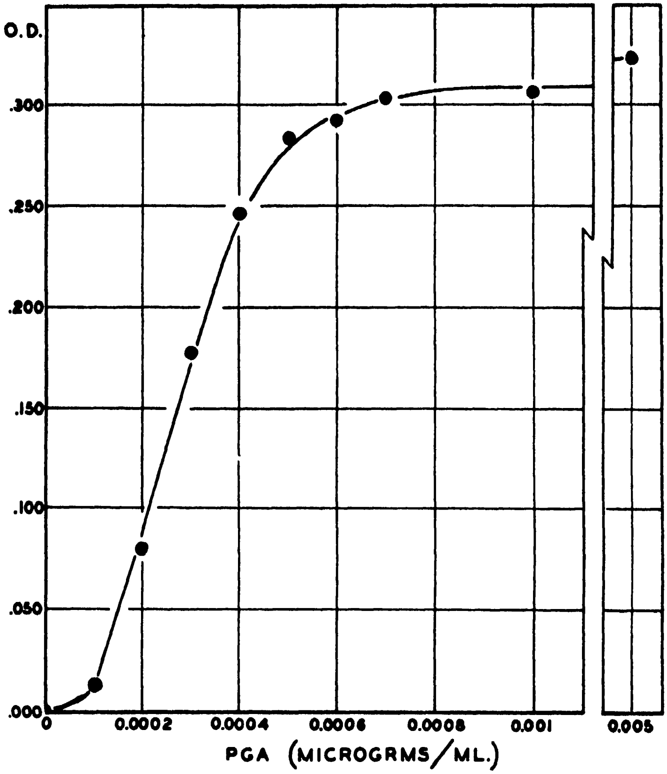

FIG. 4. Dose response to pteroylglutamic acid (PGA). Results from third serial transplant after 72 hr. incubation. In this case the yeast nucleic acid was substituted for by the following: guanylic acid, 25 γ/ml.; adenylic acid, 25 γ/ml.; cytidylic acid, 50 γ/ml.; uracil, 50 γ/ml.

DISCUSSION

The hypothesis was advanced (16) that obligatory storage of PHGA might account for the fact that its activity was higher than that of free folic acid. It was found that the activity increased in direct proportion to the number of glutamic acid residues. The present results strengthen this hypothesis, for it appears that inhibitors were present in the previously used crude "Factor II" preparations and that these inhibitors were acting on the enzymes which were responsible for conjugating the folic acid. Without the inhibitors the conjugation of PGA is rapid and efficient and the activities of PGA, PTGA and PHGA are equal (when calculated on the content of PGA present in the conjugates).

The fallacies inherent in the *L. casei* assay methods for riboflavin, pantothenic acid, and nicotinic acid, as well as for pyridoxine (9), have been demonstrated. In spite of digestion with pepsin and takadiastase (3), it now becomes apparent that not all of the vitamins were released from their bound condition in the crude extracts, for *Tetrahymena* was able to grow in what appeared to be vitamin-free medium not enriched with certain vitamins now shown to be essential growth factors. Moreover, it was shown in the earlier work that *Tetrahymena* apparently synthesized small amounts of riboflavin, pantothenic acid, and nicotinic acid. The response of *L. casei* was increased after *Tetrahymena* had grown in the media. It is probable that the actual facts are that *Tetrahymena* was utilizing the vitamins present in a bound condition and that these vitamins were made available to the *Lactobacillus* from the protoplasm of the ciliates. The ability of *Tetrahymena* to hydrolyze complex substances has been observed many times.

In the light of these results, and those previously presented on pyridoxine (9), it appears likely that *Tetrahymena* can be adapted for the assay of those B vitamins which are essential for its growth. Our experience has convinced us that the *L. casei* assay methods are unreliable where precise results are required.

SUMMARY

1. The B vitamin requirements of the animal microorganism, *Tetrahymena geleii*, were reexamined with a purified B vitamin-free base medium.
2. The nonessentiality of biotin and choline was confirmed.
3. In the present medium, it can be shown that pteroylglutamic acid and its conjugates possess the same activities for *Tetrahymena*, when calculated on the basis of free-PGA content.
4. Pantothenic acid, riboflavin, and nicotinic acid were found to be essential to the growth of this organism. Quantitative determinations are given.
5. These results again demonstrate the inadequacy of the *L. casei* assay methods.

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Chemical Studies of the Brain in Dogs with "Running Fits"[1]

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INTRODUCTION

In 1946, Erickson *et al.* (1) suggested that the convulsive seizures seen in dogs suffering from "running fits" might be related to epilepsy. These workers were able to demonstrate electroencephalographic abnormalities in dogs fed wheat gluten which were comparable to those seen in human epilepsy. Shortly thereafter, Mellanby (2) showed that wheat flour treated with NCl_3 (agene) caused "running fits" when fed to dogs.

Newell *et al.* (3) were unable to observe any significant chemical changes in the blood of dogs which had developed fits. Previous studies on dogs have shown that seizures induced by metrazole are characterized by an increased level of lactic acid and a decrease in the phosphocreatine within the cerebral tissue (4). Similar changes are associated with seizures induced by various convulsant drugs or by electrical stimulation in the cat (5). These acute changes are thought to be characteristic of the convulsive state and have been interpreted as a concomitant of the greatly increased oxidative metabolism associated with the increased energy expenditure of the cortical neurones (4).

The condition of "running fits" in the dog offers an opportunity to investigate changes in these and other cerebral constituents, which might possibly be associated with increased susceptibility to seizures.

PROCEDURES

Three healthy young mongrel dogs were used in this work. One dog was fed a ration containing 84% white flour treated with 4 g. NCl_3/cwt. After 21 days on this diet the dog showed symptoms of "running fits." The animal was continued on the toxic

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[1] This work was supported in part by a grant from the Wallace and Tiernan Company, Inc., Newark, N. J., and in part by a grant from the Wisconsin Alumni Research Foundation.

ration for 4 days following the onset of fits. It was then anesthetized with nembutal and cerebral tissue was obtained for analysis.

The other two dogs were fed a ration containing 84% whole wheat flour treated with 20 g. NCl_3/cwt. Fits were produced in both animals within 48 hr. Two days following the onset of fits, both animals were anesthetized with nembutal and cerebral tissue was obtained for analysis.

In each instance the brain was exposed in preparation for freezing *in situ*. A blood specimen was then obtained from the femoral artery and a measurement of blood pressure was made from a large needle in the same artery. Immediately thereafter, the brain was frozen with liquid air and a large area of the cortex removed to a depth of approximately 1 cm. In all animals, normal blood pressure, adequate respiration, and bright red color of the arterial blood indicated an adequate supply of oxygen to the brain at the time of freezing. The frozen specimen, weighing 5–6 g., was crushed, ground in a chilled mortar, and portions of the powdered material used for the various determinations.

Glucose was determined on $Cd(OH)_2$ filtrates of blood and brain by a method similar to that of Miller and Van Slyke (6), but differing in such details as the use of larger samples and a more strongly alkaline ferricyanide reagent. It was found necessary to correct for non-sugar reducing substances, determined after fermentation by

TABLE I

Brain Constituents in "Agenized" Dogs under Nembutal

Dog no.		1	2	3	Normal
Blood glucose		104	101	162	105 to 166[b]
Cerebral tissue					
Glucose		75	66	101	35 to 86
Glycogen		142	94	123	70 to 150
Lactic acid		4.0	13.3	18.6	9 to 26
Acid-soluble phosphorus		66.1	66.7	66.6	63 to 65
Fraction ppt. by $Ca(OH)_2$	Inorganic P	6.7	8.4	8.8	6.8 to 8.5
	Nucleotide P				
	Acid-labile	17.8	18.3	18.3	17 to 19
	Ribose monophos.[a]	9.5	9.5	9.7	8.6 to 9.7
	Residual organic P	3.3	5.6	4.6	3.7 to 4.4
Fraction not ppt. by $Ca(OH)_2$	Phosphocreatine P	12.6	9.5	9.0	9.4 to 11.1
	Acid-labile P	0.6	0.4	0.6	0.5 to 1.2
	Pentose, as ribose monophos. P[a]	0.6	0.9	1.1	0.5 to 0.7
	Residual organic P	15.0	14.1	14.5	12.3 to 14.3

Results are expressed in mg./100 g. tissue.

[a] Corrected for a small artifact.

[b] Not corrected for non-sugar reducing substances.

washed yeast cells. Glycogen was determined by the procedure of Kerr (7), with substitution of ferricyanide reduction for the copper reduction. Lactic acid was determined by a modification of the method of Barker and Summerson (8). The acid-soluble phosphorus compounds were determined by procedures previously described (9).

Results

The results of the analyses are given in Table I. With the exception of one rather low lactic acid value, the levels of the cerebral constituents determined are judged to be in the normal range by comparison with established normal values. For glucose (which must be considered in relation to its level in the blood), glycogen and lactic acid, groups of normal values are given by Kerr and Ghantus (10) and by Gurdjian *et al.* (4). For the acid-soluble phosphorus fractions normal values are given by Stone (9). Data from these sources are given in the last column of Table I.

Conclusions

Inasmuch as the cerebral constituents examined were found to be present at normal levels, it appears unlikely that there exists any chronic disturbance in the cerebral oxidative metabolism or phosphorylating mechanisms of dogs suffering from "running fits."

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Studies on the Function of Biotin in the Domestic Fowl [1,2,3]

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INTRODUCTION

Biotin may function in the synthesis of oleic acid, since this acid has been found to replace the growth-promoting action of the vitamin for several lactic acid bacteria (1,2). Biotin appears to be involved in the synthesis of aspartic acid by microorganisms (3), in the utilization of bicarbonate by bacteria (4), and in the biosynthesis of oxalacetic and α-ketoglutaric acids (5). It has been suggested that the vitamin may function in CO_2 transfer (6), and the action of biotin has been shown to be localized in oxalacetate decarboxylase, suggesting that its function may be that of a coenzyme (7).

The necessity of biotin for embryonic development in the domestic fowl has been demonstrated (8), and it has been reported that a deficiency of this vitamin in the maternal diet results in two peaks of embryonic mortality, one during the first week, and another during the last 3 days of the incubation period (9). Congenital perosis and ataxia as well as characteristic skeletal deformities develop when hens are fed a low biotin diet which does not favor the intestinal synthesis of the vitamin (9,10).

The present investigation was initiated to study the relation of fat to biotin metabolism in the laying fowl, to determine the critical period at which it is actually utilized in development, and to study the physiological mechanisms through which a deficiency of the vitamin may be expressed.

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[1] Supported in part by a grant from the Western Condensing Company, San Francisco, California.

[2] We are indebted to Wilson Laboratories, Chicago, Illinois, for the liver fraction L; to Merck & Co., Rahway, N. J. for the biotin.

[3] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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EXPERIMENTAL

The management and experimental procedure was the same as that outlined by Couch *et al.* (10). The pullets were fed an all-mash laying ration for 3 weeks prior to the start of the experiment, at which time the birds were divided into one group of 10 (Diet B31), one group of 6 (practical all mash diet), and three groups of 4 each (Diets B34, B38, and B39).

The composition of the basal diet (B31) was as follows: sucrose 63; purified casein 18; gelatin 5; salts IV 5; liver fraction L 4; fish oil (3000 A-400 D) 2; soybean oil 3; choline chloride 0.2; crystalline vitamins were added as follows/kg. of diet; thiamin HCl 4; riboflavin 6; Ca pantothenate 15; niacin 100; 2-methyl-1,4-naphthoquinone 0.5; pyridoxin HCl 4; α-tocopherol 3. The addition of 0.2 mg. of biotin/kg. to diet B31 gave B34. Diets B38 and B39 were identical to diets B31 and B34, respectively, except that soybean oil was omitted and the fish oil was replaced by fortified haliver oil (60,000 A-10,000 D-2 drops per hen daily).

Eggs were set aside for biotin, fat, and iodine number determinations on the last two days of each week. A modification of the method of Wright and Skeggs (11), in which turbidity served to indicate the growth of *Lactobacillus arabinosus*, was used to determine the biotin content of the eggs. The fat content of the egg yolk was determined by acid hydrolysis according to the method of the Assoc. Offic. Agr. Chemists (12). The egg yolk fat sample was dried *in vacuo* at 55°C., and the iodine number of this fat was determined by the Hanus method (12).

Representative 10-day embryos from hens fed the low biotin diet (B31) and from those fed diet (B34) were removed from the incubator and freed of extraembyronic membranes and the yolk sac. Three embryos were ground with sand, extracted with trichloroacetic acid and analyzed for citric acid by the method of Perlman, Lardy and Johnson (13). Similar determinations were made on leg bones (femur and tibiotarsus) of day-old chicks from hens fed the diets indicated above.

The fixation of CO_2 by a day-old chick exhibiting the symptoms of biotin deficiency and a normal chick was studied by injecting intraperitoneally radioactive carbonate.[5] One hr. after this injection, the birds were skinned, weighed, immersed in 6 N HCl in open flasks and autoclaved for 2 hr. at 15 lbs. pressure. After concentration *in vacuo*, radioactivity measurements were made on samples of the whole hydrolyzate by means of a Geiger counter.

A study was made to determine whether the muscles or the nerves were affected by a deficiency of biotin in the day-old chick. Chicks which exhibited all gross pathological symptoms of biotin deficiency were selected and divided into 2 groups. Each chick in the first group was given an intraperitoneal injection of 0.2 or 0.4 unit of curare, a drug which blocks the motor impulses. In the second group, the sciatic nerves of each chick were severed and this was followed by cutting the spinal cord in the lumbar region; muscles of the tibiotarsus were severed next about the median line and finally the gastrocnemius tendons were cut.

Following the experiment on the relation of fat to biotin metabolism the 10 hens were continued on the low biotin diet (B31) for 6 months. The per cent hatchability of eggs from these birds at the start of the latter period was zero. All eggs laid were used for injection studies described below. All injections were made into the albumen with a syringe and a 26 gauge needle through a small hole drilled in the large end of the egg. The holes were sealed with collodion. The eggs (produced each week) were divided into two lots. The eggs in one group were each injected with 0.1 cc. of water (pH 8.0–9.0); this group served as the negative control. Each egg in the second lot was injected with 2 γ of biotin in 0.1 cc. of water. This procedure was repeated with 10 and 25 γ of biotin per egg. Likewise, 25 γ of biotin was injected at 0, 48, 72, 96, and 120 hr. of incubation into individual eggs according to the procedure outlined above. In addition, one test was conducted where 2 mg. of aspartic acid in 0.1 cc. of water was injected into each egg with an equivalent amount of water as a negative control. Approximately 50 eggs were used in each of the treatments outlined above.

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[5] Supplied through the courtesy of Dr. R. H. Burris, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin.

RESULTS AND DISCUSSION

This investigation has shown that unsaturated fats containing oleic acid (fish oil and soybean oil) do not have any apparent effect on the egg production, hatchability, or biotin content of the eggs from hens fed diets low in biotin or those containing an ample quantity of the vitamin (Fig. 1 and 2). The per cent egg production of hens fed diets B31, B34, B 38, B39, and the practical all-mash diet was approximately the same until the last week of the test when there was a slight decrease in the production of those birds fed the diets low in fat. The per cent hatchability of eggs from hens fed low biotin diets (Fig. 1) was similar to that

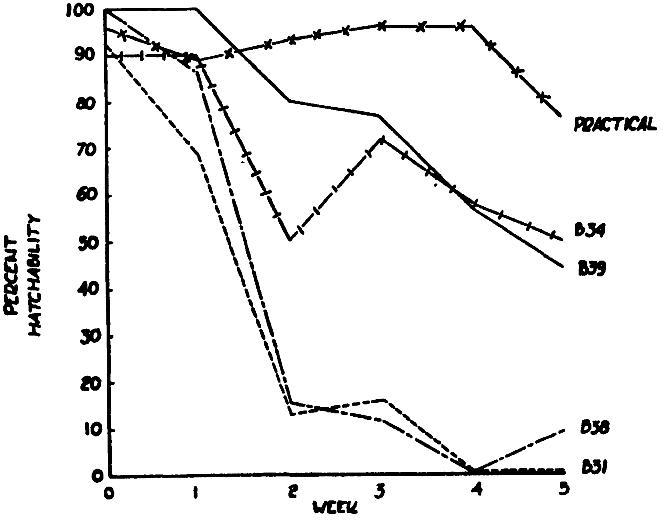

FIG. 1. Effect of biotin and fat on hatchability.

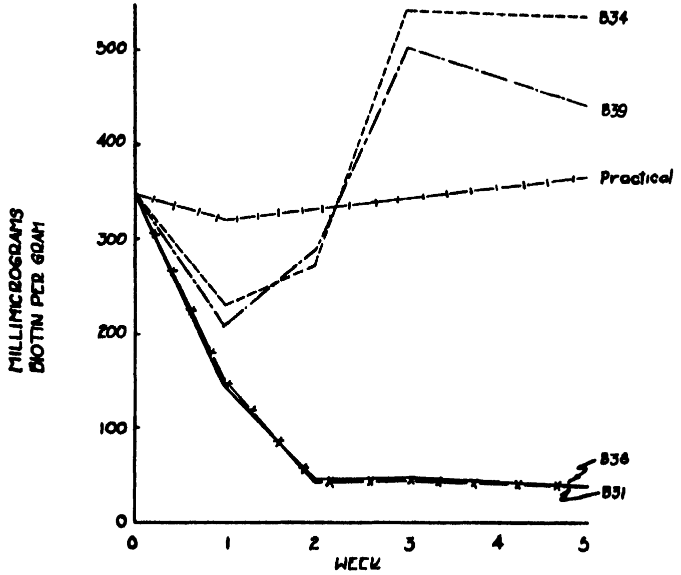

FIG. 2. Relation of biotin and fat to the biotin content of egg yolk.

reported by Couch *et al.* (14), and did not appear to be influenced by unsaturated fats in the diet. Hatchability of eggs from hens fed 0.2 mg. of biotin/kg. of diet was significantly higher than that of those fed the low biotin diets (Fig. 1), but again did not appear to be influenced by unsaturated fat in the diet. The biotin content of the egg yolks from hens in this study is expressed in mγ/g. on a fresh weight basis (Fig. 2). It may be noted that egg yolks from hens fed 0.2 mg. of biotin/kg. contained more of the vitamin than did those fed the low biotin diets; these values were not influenced by the feeding of 2% fish oil and 3% soybean oil. The decrease in the biotin content of the egg yolks from hens fed diets low in the vitamin parallels the decrease in hatchability described above (Figs. 1 and 2). The biotin content of the whites was determined and the same general trends were observed as indicated for the egg yolks.

These results are somewhat in disagreement with those of Trager (15) in which it was reported that a fat-soluble fraction (FSF) from plasma injected into chicks would protect against egg white injury. This factor was later shown to be oleic acid (16). Soybean oil contains 25–30% oleic acid and has an iodine value of 115–145 (17); the iodine value of cod liver oil is 177 (17). From Trager's work it was thought that omission of these fats from the low biotin diet might produce a more severe deficiency than that obtained with 2% fish oil and 3% soybean oil in the diet. It was also thought that omission of fat from the diet containing 0.2 mg. of biotin/kg. of diet might result in a decrease in the biotin content of the egg, thus producing a lower hatchability than was obtained with the same level of the vitamin and 5% unsaturated fat. Attention is also called to the fact that egg yolk fat contains 47–51% oleic acid (18). It should be pointed out that Trager's experiment was carried out by the injection of FSF into the chicks while in the present investigation the fats were mixed in the diet, consumed by the birds, and carried through the normal process of digestion.

Biotin did not appear to have any appreciable effect on the fat content of the eggs or the degree of saturation of the fat as measured by the iodine values. However, the egg yolk fat was more saturated and had a lower iodine number when the hen had to make this fat from a carbohydrate source (sucrose) than when 2% fish oil and 3% soybean oil was included in the diet. The iodine numbers of egg yolk fat reported in this study for eggs from hens fed the practical diet are within the range (80–84) of those reported by Cruickshank (18), in which hens were fed diets composed of naturally-occurring feeds. Cruickshank was able to increase the unsaturation of egg yolk by feeding linseed oil, and obtained an iodine value of 123, but was not able to alter the iodine number of egg yolk fat by including 28% mutton fat or palm kernel oil in the diet. In the present study, the iodine numbers of egg yolk fat from hens fed a purified diet, which did not contain any added fat, were decreased (64.0) below those of hens fed a similar diet containing 5% fat (77.0), or those from hens fed a practical diet (82.0).

In view of the various functions which have been proposed for biotin, citric acid determinations were made on 10-day embryos and on the leg bones of day-old chicks with the possibility that information might be obtained on the function of the vitamin in this species. The citric acid content of 10-day embryos from eggs laid by hens fed the low biotin diet and those fed 0.2 mg. of biotin during the first and second weeks of the present investigation was 11–12 mg./100 g. These citric acid values are approximately the same as those reported by Dickens (19) for 8–12 day embryos. The citric acid content of the leg bones (femur and tibiotarsus) of day-old chicks from hens fed the low biotin diet, from those fed 0.2 mg. of biotin/kg. of diet, and from those fed the practical all-mash diet during the first and second weeks of the test was 81–92 mg. of citric acid/100 g. of bone, and did not appear to be related to the biotin content of the hen diet.

Identical Geiger counts were obtained on samples of whole hydrolyzate of a biotin-deficient and a normal chick, both of which had been injected with an equivalent amount of radioactive carbonate 1 hr. prior to killing and autoclaving, This indicates that the uptake of CO_2 had proceeded at the same rate in both cases.

From results of techniques employed in this study it has not been possible to demonstrate that biotin functions in the fowl in a manner similar to that observed for microorganisms with regard to oleic acid replacing biotin (1,2). Work with microorganisms has indicated that biotin functions in CO_2 transfer (4,5,6,7); this function of the vitamin could not be demonstrated with the chick, either through citric acid analyses of embryos and bones of day-old chicks or by radioactivity studies under conditions of the experiments conducted. However, it should be pointed out that the chick is a much more complex organism than the bacteria which have been used in studying the function of biotin, and the vitamin probably has as yet undiscovered functions in the chick.

Injections of 0.2 and 0.4 units of curare failed to relieve the continuous rotational movements of the head (ataxia) or any other biotin deficiency symptoms over a period of 12–18 hr. The larger dose of the drug was probably approaching the lethal limit, since one of the chicks died within 10 min. after 0.4 unit of curare was injected. Severing of the sciatic nerves, spinal cord (lumbar region), and leg muscles failed to relieve the stiff hocks and/or flexed or extended toes in any instance. The latter was finally relieved by severing of the gastrocnemius tendon. These observations indicate that the nerves and muscles are probably not affected in the biotin deficient chick but that the pathological state lies in the gastrocnemius tendon. It might also be mentioned that perosis is one characteristic symptom of biotin deficiency in the growing chick.

The per cent hatchability of eggs from hens fed the low biotin diet and injected with 2, 10, and 25 γ of biotin per egg is shown in Fig. 3. The critical level of biotin for hatchability under these conditions is in the vicinity of 10 γ of the vitamin per egg; one chick from an egg injected with this quantity of the vitamin had a parrot beak, a shortened and twisted tibiotarsus, and a very much shortened tarsometatarsus.

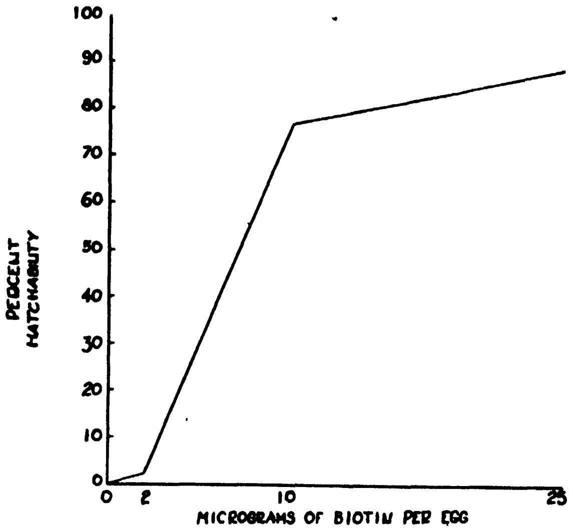

FIG. 3. Effect on hatchability of injecting various quantities of biotin into biotin-deficient eggs.

These symptoms are characteristic of embryos from eggs laid by hens fed a low biotin diet. The injection of 10 γ of biotin per egg discussed above, is about 5 times the quantity of biotin previously reported as being adequate for embryonic development (14). However, in the latter case, the biotin was in the egg in its naturally occurring state, whereas in the present investigation it was injected into the white of the egg which contains avidin. The increased embryonic requirement for biotin in the case of the injected eggs is possibly due to the fact that a portion of the injected biotin may combine with avidin and not be available to the embryo or that biotin in its naturally occurring bound form is more readily utilized by the developing embryo.

The relationship between time of injection of 25 γ of biotin per egg and hatchability is indicated in Fig. 4. The per cent hatchability decreased from 89 for eggs injected at the start of the incubation period to zero for those injected with 25 γ of biotin at 120 hr. A very severe perosis was observed in about 50% of the chicks hatched from eggs injected with the vitamin at 96 hr. of incubation. This was characterized by slipping of the gastrocnemius tendon and complete displacement of the proximal end of the tarsometatarsus. This observation indicates that biotin enters into some reaction during embryonic development of the chick, which becomes critical with regard to perosis and gastrocnemius tendon metabolism at about 96 hr. of incubation. However, from the large percentage of embryos which die at approximately 72 hr., it is indicated that biotin enters into other reactions not elucidated in the present investigation.

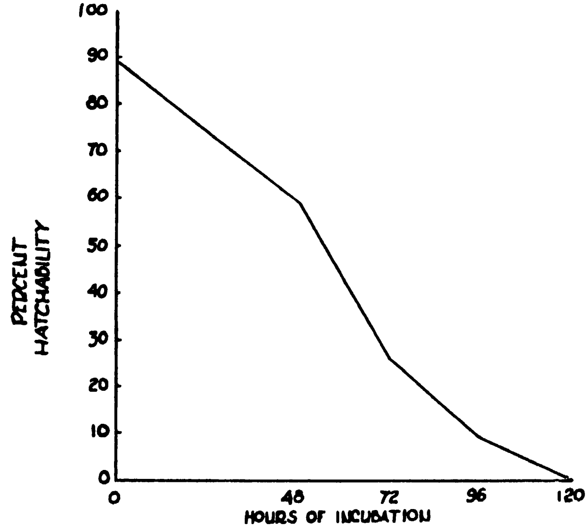

FIG. 4. Relation between time of injecting 25 γ of biotin into biotin-deficient eggs and hatchability.

Aspartic acid did not replace biotin to the slightest extent when injected in 2 mg. amounts into low biotin eggs prior to the start of the incubation period; no live chicks were obtained and the same peaks of mortality were obtained as described below for eggs which were used as negative controls. Biotin has been shown to be necessary for the synthesis of aspartic acid by microorganisms (3). In the case of the developing chick embryo the vitamin may not be required for this purpose. However, aspartic acid is found in the proteins of the egg.

No live chicks were obtained from eggs injected with 0.1 cc. of water and used as negative controls in this study. Two distinct peaks of embryonic mortality were observed in the water-injected eggs. One of these occurred about the third day and the latter near the end of the incubation period. This confirms the work of Cravens, McGibbon and Sebesta (9) in which similar peaks of embryonic mortality were observed in eggs from hens fed a low biotin diet.

SUMMARY

1. Unsaturated fats (soybean oil and cod liver oil) did not appear to have an appreciable effect on egg production, hatchability, biotin content of egg yolks and whites, fat content of egg yolk, or the iodine number of egg yolk fat when hens were fed diets low or high in biotin, with or without 5% fat.

2. The iodine values of egg yolk fat from hens fed low fat diets were lower than those from hens fed the same diets with 5% fat, or from those fed the practical diet.

3. The citric acid content of 10-day embryos and leg bones of day-old chicks did not appear to be related to the biotin content of the maternal diet.

4. The CO_2 uptake of biotin-deficient day-old chick was the same as that of a normal one.

5. Approximately 10 γ of biotin is required for an embryo from a hen fed a low biotin diet when the vitamin is injected into the egg.

6. When biotin-deficient eggs were injected with the vitamin, hatchability was 89% for those injected at the beginning of the incubation period and decreased in a linear relationship to zero for those injected at 120 hr. of incubation.

7. A very severe perosis was observed in chicks from eggs injected at 96 hr. This indicated that the vitamin is required for some reaction which becomes critical with regard to gastrocnemius tendon metabolism at about 96 hr. of incubation.

8. There are probably other functions of biotin which are not related to tendon metabolism, and which are not elucidated in the investigation reported herein.

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The Synthesis of Niacin from Tryptophan in Rat Liver Slices [1]

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INTRODUCTION

Following the observations by Krehl *et al.* (1) that tryptophan, as well as niacin, could relieve the symptoms of nicotinic acid-deficiency, and the demonstration the same year by Rosen and associates (2) that the addition of tryptophan to the diet or the parenteral injection of this amino acid caused a marked increase in the production of niacin in the rat, considerable interest has been aroused regarding the site of this synthesis.

Krehl *et al.* (1) reported that the growth-inhibitory effects of low-tryptophan, low-niacin diets could be produced on non-corn diets when the sugar was sucrose. This effect could be overcome, however, not only by the feeding of tryptophan or niacin, but also by the administration of a carbohydrate which produced a favorable intestinal flora for the synthesis of niacin. Ellinger *et al.* (3,4) suggested that in man the flora of the intestinal tract may be able to synthesize niacin which can subsequently be absorbed by the gut. This theory was based on the observation that human subjects on a constant diet excreted less N^1-methylnicotinamide (the principal end-product of niacin metabolism) after receiving dosages of sulfonamides. The excretion of the methylated niacin rose again upon cessation of treatment.

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[1] Contribution No. 183 of the Department of Biochemistry and Nutrition.

[2] Major, United States Air Forces. The work is presented in partial fulfilment of the requirements of the degree of Master of Science in Biochemistry. The authors wish to acknowledge the use of the facilities of the Hancock Foundation.

The influence of pyridoxine on tryptophan metabolism was discovered by Lepkovsky (5) when he observed that the urine of pyridoxine-deficient rats, when treated with ammonium sulfate, yielded a green pigment. This constituent of pyridoxine-deficient urine was later identified as xanthurenic acid, a known metabolite of tryptophan. Schweigert and Pearson (6) showed that pyridoxine-deficient rats could not convert tryptophan to niacin or to N^1-methylnicotinamide as well as rats fed pyridoxine. Rosen, Huff and Perlzweig (7) also demonstrated that prolonged pyridoxine deficiency in young animals adversely affected the transformation of tryptophan to niacin in the rat. This metabolic defect was not restored to normal, even after two weeks of pyridoxine feeding. This slow return to normal suggested an effect due to an altered intestinal flora. If, however, the intestinal flora are the major source of niacin in the rat, the extremely rapid response to parenteral injections of tryptophan (Rosen *et al.* (2)) is hard to explain. In fact, Ling (8) has recently noted that the increase in DPN content of the erythrocytes may occur within 4 hr. after the injection of tryptophan. Bell and coworkers (9) were unable to show a correlation between the excretion of N^1-methylnicotinamide and the degree of pyridoxine deficiency.

EXPERIMENTAL

The rats used in these experiments were of the University of Southern California strain. They were kept in large wire cages with screen bottoms to minimize coprophagy. The animals were divided into two groups of 40 each; one group received no pyridoxine in the diet, the other group received 20 mg. pyridoxine hydrochloride/kg. of diet. In accordance with the findings of Schweigert *et al.* (10), a high protein diet (50%) was used. Schweigert found that a high protein diet increased the hepatic storage of pyridoxine on a diet containing a sufficiency of the vitamin, and at the same time lowered the hepatic pyridoxine level on a diet containing no vitamin B_6. The animals showed signs of pyridoxine deficiency within 3 weeks. During the 14 weeks following this period, the animals on pyridoxine-deficient and normal diets were sacrificed periodically and the livers used for the tests.

The composition of the basal and experimental diets is given in Table I.

Liver slices of a uniform thickness were incubated with various substrates, and assayed for the production of niacin by the micro bioassay procedure (11). To provide maximum surface area for oxygenation, the slices were placed in a 25 ml. Erlenmeyer flask with 2 ml. of substrate and were shaken for 2 hr. in a water bath at 37°C. The substrates contained 0.2 ml. of 10% glucose, 1 ml. of $M/15$ PO_4 buffer ($pH = 7.4$), and 0.01 M L-tryptophan in Ringer's solution to give appropriate amounts of tryptophan. Additional Ringer solution not containing tryptophan was added to bring the total volume to 11.2 ml. An atmosphere of pure oxygen was maintained as nearly as possible by frequent oxygenation. The period of 2 hr. was chosen to minimize the error due to the growth of bacteria in the substrate.

TABLE I

Composition of the Experimental Diets

	per cent
Casein (vitamin free)	50.0
Sucrose	36.4
Celluflour	4.0
Salts (Osborne and Mendel)	4.0
Cottonseed Oil (Wesson)	3.0
Biotol (concentrate of vitamins A and D)	2.0
L-Cystine	0.2
Choline hydrochloride	0.3
	mg./kg. diet
Thiamine	20
Folic acid	5
Riboflavin	20
Calcium pantothenate	40
Pyridoxine hydrochloride	20 (control diet only)

Immediately upon removal from the shaker, H_2SO_4 was added to bring the concentration to 1N and the contents hydrolyzed in the autoclave at 20 lbs. pressure for 1 hr. The samples were then adjusted to pH 6.8 with brom-thymol-blue.

As one check on the method, 8.5 γ of niacin were added to the Ringer's solution substrate; in all cases the niacin could be demonstrated in the assay as unchanged. Control tests were also made, in which the medium was acidified before addition of the liver slices. These were autoclaved at the same time as the test samples. The tryptophan-containing substrates were also acidified, autoclaved, and analyzed by the micro bioassay method and were found to contain nothing which would stimulate the growth of Lactobacillus arabinosus.

RESULTS

The average gain in weight of the rats in each group is shown in Fig. 1. The typical symptoms of edema and acute erythema involving the snout, paws, ears, and tail were noted within 2–3 weeks. No pathological symptoms appeared in the control group at any time.

Experiments were carried out on liver slices obtained from nine rats. Four of these animals were suffering from pyridoxine deficiency while the remaining 5 rats were control animals. A typical experiment of each series is reported in Table II.

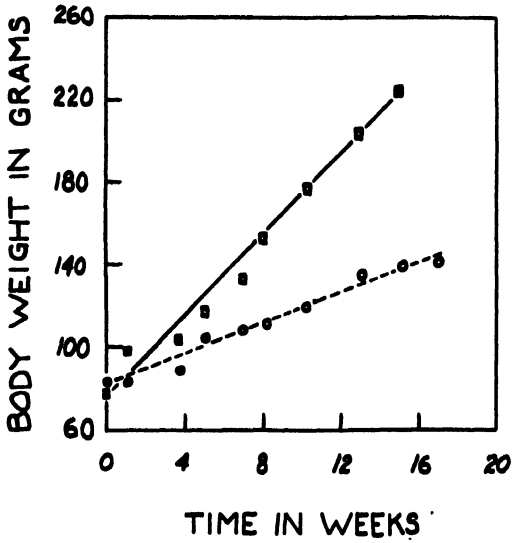

FIG. 1. Average body weight of the pyridoxine-deficient rats (dashed line) and of normal control rats (solid line).

Of the 4 tests on livers from pyridoxine-deficient rats, one showed a definite augmentation of the niacin content when incubated with tryptophan. Another test increased the niacin present by about 25%. Of the 5 normal rat livers, 4 also gave positive response to tryptophan by an increased niacin formation. It is probable that the negative responses noted might be attributed to the destruction of the enzyme system prior to the completion of the tests. The average niacin content of the livers, together with the increase caused by incubation with tryptophan, is included in Table III. The average recovery of added niacin is also recorded in this table.

TABLE II

Showing a Typical Experiment with Liver Slices from a Pyridoxine-Deficient and a Normal Rat

Flask no.	Tryptophan added	Incubated liver				Unincubated livers			Per cent increase in niacin
		Liver slice weight	Niacin found in γ			Liver slice weight	Niacin found in γ		
			Total	Per mg. liver	Average		Total	Per mg. liver	
Pyridoxine-deficient rat (Experiment no. 4)									
	mg.	*mg.*				*mg.*			
1	0	102	9.1	.089	0.092	102.5	10.3	0.101	−8.9
2		101	9.5	.094					
3	1.8	101.5	18.8	.185	.177	97.5	11.4	.117	51.4
4		101	17.1	.169					
5	0.9	99.5	13.7	.138	.137	101.5	9.2	.091	50.5
6		100	13.6	.136					
7	0.18	100.5	13.7	.136	.134	99	10.1	.102	31.4
8		104	13.3	.132					
9[a]	0	103.5	18.6	.180	.175	102	17.5	.172	1.7
10		97.5	16.5	.170					
Normal rat (Experiment no. 10)									
1	0	104	13.3	.128	.129	104	13.3	.128	0.8
2		103	13:3	.129					
3	1.8	97.5	17.7	.182	.183	104.5	14.1	.135	35.5
4		98	18.0	.184					
5	0.9	99	19.4	.196	.185	105	13.6	.130	42.4
6		94.5	16.3	.173					
7	0.18	100.5	16.0	.152	.163		No assay		
8		96.5	16.8	.174					
9[a]	0	104	23.9	.229	.233	97.5	22.4	.230	1.3
10		101	23.9	.236					

[a] 8.5 γ niacin added to each of these flasks immediately before analysis.

TABLE III

The Average Niacin Content of the Livers of Rats on a Normal or Pyridoxine-Deficient Diet before and after Incubation with Tryptophan

Normal rats							Pyridoxine-deficient rats						
Expt. no.	Niacin γ/g. liver	Extra niacin/g. of liver			Recovery of added niacin		Expt. no.	Niacin γ/g. liver	Extra niacin/g. of liver			Recovery of added niacin	
		A	B	C	γ	Per cent			A	B	C	γ	Per cent
5	123	37	40	17	9.8	115	1	83	3	10	42	8.5	100
6	120	32	9		6.4	75	2	80	23	15	10	9.2	107
7	127	9	5	2	7.9	93	3	77	4	2	−1	9.6	113
9	137	14	8	−4	9.8	115	4	92	85	45	40	8.3	98
10	129	52	54	34	10.0	118							
Av.	127	29	23	12	8.8	103	Av.	84	29	18	23	8.9	104

The following amounts of tryptophan were present/ml.: A, 1.8 mg.; B, 0.9 mg.; and C, 0.18 mg.

DISCUSSION

The data indicate that the liver is one site of synthesis of niacin in the albino rat. There is no indication that the synthesis proceeds any more effectively in the normal rats than in those animals which had been deprived of pyridoxine over a long period of time. The average extra niacin in the samples where the substrates contained tryptophan at several levels was 29, 23, and 12 γ/g. of liver for the normal rats compared with 29, 18, and 23 γ, respectively, for the rats which had been on the pyridoxine-deficient diet. The recoveries of added niacin approximated 100% in each series which included one low value. These data would seem to indicate that niacin synthesis may proceed during pyridoxine deficiency. However, it should be pointed out that there can be no absolute pyridoxine deficiency in the living rat, so these results do not entirely preclude the fact that pyridoxine may play a role in the synthesis of niacin from tryptophan.

In spite of the fact that the synthesis of niacin undoubtedly does occur in the liver of the pyridoxine-deficient rat, there is considerable evidence that the basal level of niacin in the livers of the rats fed the normal diet is markedly elevated over those on the pyridoxine-deficient diet. Thus, in the former case the values averaged 127 γ (range, 120–137)/g. liver, and in the latter instance 84 γ (range 77–92)/g liver. These data confirm the previous reports (6,9) that pyridoxine facilitates the conversion of tryptophan to niacin.

One possible explanation for the fact that two of the livers tested (one each from the normal and pyridoxine-deficient group) seem to have lost their ability to synthesize niacin from tryptophan is suggested from the work of Perlzweig (13). Perlzweig observed that, when nicotinamide was incubated with liver slices, it was converted to N^1-methylnicotinamide. Free nicotinic acid could not be methylated by this procedure. This suggests that the normal pathway of tryptophan metabolism would be through nicotinamide to N^1-methylnicotinamide, and that positive results as measured by the micro bioassay would occur only when the enzyme concerned with methylation had been poisoned or was absent. Unfortunately, the analyses are not available in the present series for changes in the N^1-methylnicotinamide.

The data cannot be used as proof that liver is the *sole* site of synthesis The work by Ellinger (3,14), on the effects of sulfonamides on niacin excretion, and by Krehl (12), on the effects of carbohydrates in the diet, show that intestinal flora may be involved.

CONCLUSIONS

1. The liver is one site of synthesis of niacin form tryptophan in the rat.
2. Pyridoxine plays a role, either directly or indirectly, in maintaining the basal level of niacin in rat liver.
3. The livers of pyridoxine-deficient rats retain the ability to synthesize niacin from tryptophan.

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A Colorimetric Modification of the Willstätter Method for the Rapid Determination of Amylase Activity in Pharmaceutical Preparations

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INTRODUCTION

Various methods have been proposed for the determination of amylase activity of pancreatin or pancreatic preparations. These are of two main types; namely, (1) the so-called "liquefaction or dextrinization" methods, which measure the power of the enzyme to completely convert a known amount of starch into products which no longer give the characteristic color reaction with iodine (12,13,14,21), and (2) the "saccharification" methods, which measure the amount of reducing sugar produced by the enzyme when acting upon an excess of starch under standard conditions (5,6,9,19,20). In the saccharification methods, the amount of reducing sugar produced has been determined either by means of alkaline copper solutions, or by means of iodine oxidation procedures. In general the Willstätter *et al.* method (20) has been found most satisfactory for our studies on amylase activity. However, a colorimetric method was desirable which would eliminate the time involved in the iodine titrations and permit the determination of a large number of samples simultaneously. It was also desirable to use a colorimetric method which would give results that could be used in the Willstätter *et al.* equation to calculate the units of amylase activity. This would allow comparisons to be made between the activities of various preparations as determined by either method.

The method outlined below is a rapid colorimetric method based on the methods developed by Sumner (15,16,17,18) for the determination of reducing sugars formed in the determination of invertase and β-glucosidase activities, using the dinitrosalicylic acid sugar reagent. The senior author had observed, while working with Sumner, that this method could also be adapted to the determination of reducing substances in the presence of starch, provided certain precautions were taken. It therefore seemed possible to use a modification of this method for the determination of amylase activity in pharmaceutical preparations in such a manner that the results could be reported in terms of Willstätter units.

Since this work was undertaken, Meyer and his collaborators (2,7,8, 10) have indicated that they have also used a modification of this procedure for determining amylase activity in their studies on crystalline pancreatic amylase.

The method outlined has the advantages that it is easy to manipulate and is less time consuming as compared with the method of Willstätter *et al.* The reagents are all stable, the color produced is stable and the method is as accurate as the Willstätter *et al.* method. The results are expressed in the same units as the Willstätter *et al.* method.

REAGENTS

Starch Solution. A 1% starch solution was prepared with U. S. P. reference standard potato starch. The moisture content was determined by drying a sample of the starch for 4 hr. at 120°C. One g. of the oven-dried starch was suspended in 10 ml. of cold distilled water. This suspension was then poured into 90 ml. of boiling water and the solution thoroughly mixed. The solution was boiled an additional 5 min. with constant stirring. The volume was then adjusted to 100 ml. by addition of distilled water to replace that lost during the heating process. Fresh starch solutions were used in each set of determinations.

Phosphate Buffer. A phosphate buffer was prepared by mixing 5.1 ml. of 0.2 *M* KH_2PO_4 with 4.9 ml. of 0.2 *M* Na_2HPO_4 which gives a pH of approximately 6.7.

Sodium Chloride Solution. A 0.2 *M* solution of sodium chloride was used to activate the enzyme.

Enzyme Solutions. Dilute enzyme solutions were prepared by suspending pancreatin in distilled water to give a final concentration of 0.2–1.0 mg./ml.

Dinitrosalicylic Acid Quantitative Reagent (16,17). The dinitrosalicylic acid reagent is prepared by adding 880 ml. of 1% 3,5-dinitrosalicylic acid solution and 255 g. of Rochelle salt to 330 ml. of 4.5% NaOH. To 10 g. of crystalline phenol add 22 ml. of 10% NaOH. Water is added to dissolve the mixture and the solution diluted to 100 ml. To 69 ml. of this solution add 6.9 g. of sodium bisulfite. Add this solution to the dinitrosalicylic acid solution and mix until all the components have dissolved. Three ml. of the reagent should contain the equivalent of 5–6 ml. of 0.1 *N* NaOH when titrated with a standard acid using phenolphthalein as an indicator. The solution should be stored in a tightly stoppered, well-filled bottle. The reagent is stable at least one year.

PROCEDURE.

Place in a 100 ml. volumetric flask 25 ml. of starch solution, 10 ml. of phosphate buffer and 1 ml. of NaCl solution. Mix thoroughly and place in a constant temperature bath at 37°C. Allow to reach constant temperature and then add 1 ml. of the enzyme solution. After exactly 10 min., add 2 ml. of 1 *N* HCl to inactivate the enzyme and stop the reaction. Add 6 ml. of 1 *N* NaOH to make the solution alkaline. Dilute the solution to 100 ml. and mix thoroughly. With a Folin-Ostwald pipette, place 1 ml. of the enzymatic digest in a clean Folin-Wu sugar tube. Then add 3 ml. of the dinitrosalicylic acid reagent. The contents of the tube are mixed by shaking the tube at an angle of about 30° to the horizontal.

Immerse the tubes in boiling water for exactly 5 min., and then transfer them to a cold bath at 5°C. Immediately dilute to 25 ml. with cold distilled water (5°C.). The solutions are then read in a colorimeter at 525 mμ and the mg. of sugar present in the solution determined by referring to a standard curve.

EXPERIMENTAL

The accuracy of the dinitrosalicylic acid test for the determination of the quantity of reducing substances produced by the enzymatic hydrolysis of starch depends largely on observing a few fundamental precautions. The method depends on the reduction of the dinitrosalicylic acid reagent by the reducing carbohydrates to the corresponding amino derivative accompanied by a change in color. This change in color can be used as a measure of the quantity of reducing substances in a test aliquot.

In the enzymatic digest, dextrins and sugars represent the reducing substances present (3). In the Willstätter *et al.* method, the number of reducing groups are expressed in terms of mg. of maltose, the principal sugar produced. The dextrins and maltose are substances which can be easily hydrolyzed to simpler compounds with an increase in the number of reducing groups. To determine whether these hydrolyzable substances could be determined by the dinitrosalicylic acid method in the presence of starch, two series of maltose solutions were prepared. Both series contained the same concentration of standard reference maltose while one series contained starch in addition to the maltose. Fig. 1 indicates that maltose can be determined in the presence of starch if the test solutions are heated for 5 min. and low concentrations of the enzyme are used. In an enzymatic hydrolysis digest, moreover, there will be re-

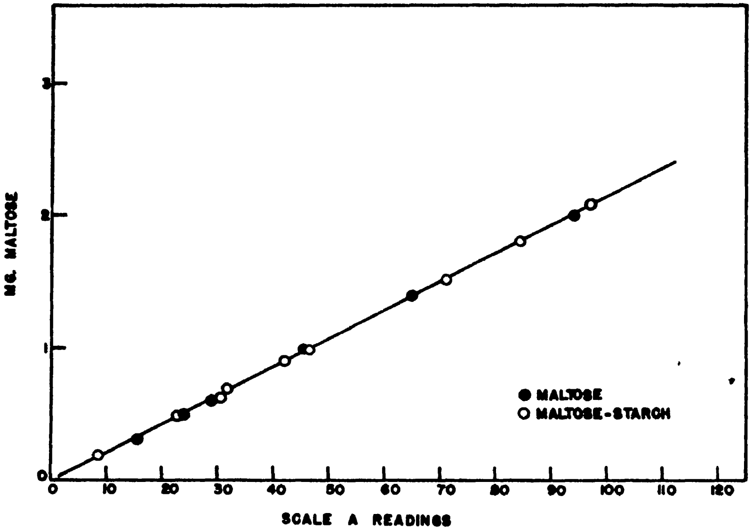

FIG. 1. Determination of maltose in the presence and absence of starch by the DNS method.

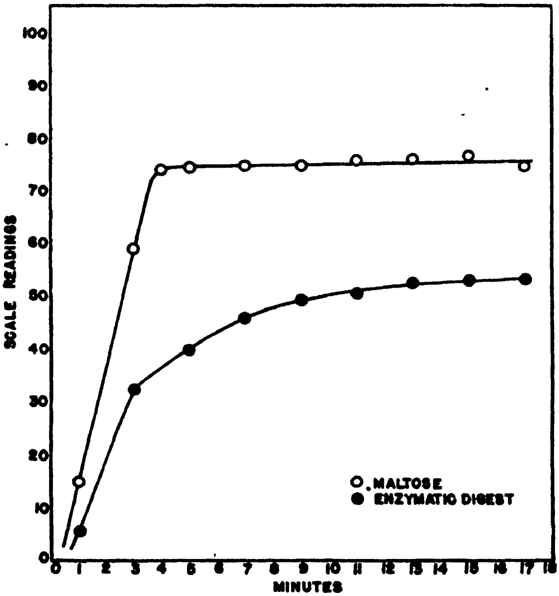

FIG. 2. The effect of heating on the scale reading of samples of maltose and an aliquot of enzymatic digest of starch.

ducing dextrins present with the maltose and starch. These dextrins will also reduce the DNS reagent when the sample is heated. With maltose samples containing starch, maximum color intensity was obtained with a 5 min. heating period (Fig. 2). With aliquots of the enzyme digests, however, heating periods of 15 min. or more had to be used to obtain a more or less constant maximum color intensity (Fig. 2). In this case, the sugars and dextrins both reacted with DNS reagent and the starch and dextrins were slowly hydrolyzed, due to alkali present in the reagent, to yield other reducing substances.

A comparison was made between the scale reading on the colorimeter for the DNS method and the number of reducing groups as calculated in mg. of maltose present in each digest as determined by the use of the Willstätter *et al.* method (19,20) in order to determine whether a relationship existed between the scale readings and mg. of maltose present. A series of enzymatic digests were prepared and aliquots used for the DNS method in which the time of heating was varied. It was found that a 5 min. heating period gave scale readings which corresponded to the mg. of maltose present.

The standard curve used in the DNS method for converting scale readings to mg. of maltose was prepared by determining the scale readings for a series of determinations in which the mg. of maltose present in the test solution has been calculated by the Willstätter *et al.* method (see Fig. 3).

As the time of heating is important in this test, the length of time the tubes stand after the heating period will also be important. As long as the tubes remain hot, reduction of the DNS reagent will continue. If the tubes are immediately immersed in cold water (5°C.) after heating, no significant increase in color intensity will result

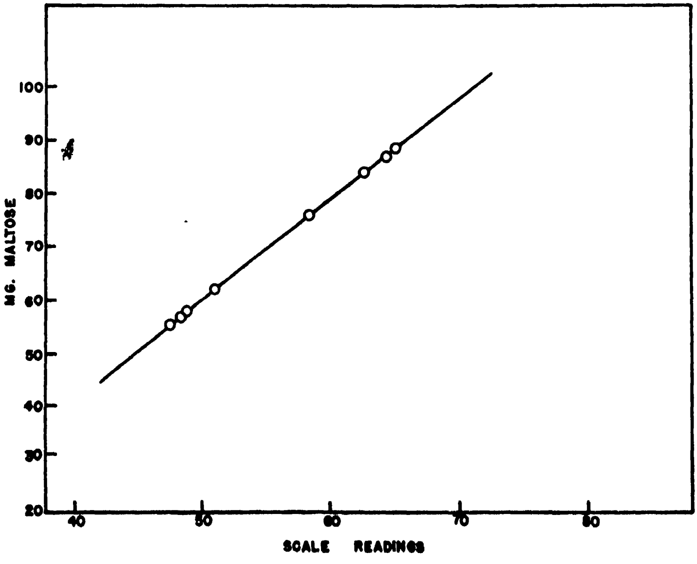

FIG. 3. Standard curve for converting scale reading for 1 ml. aliquot of the enzyme digest to mg. of maltose present in the total sample.

(see Table I). To insure complete stoppage of the reaction the tubes were transferred to the cooling bath and immediately diluted with cold distilled water (5° C.) to 25 ml.

The color developed in this test is very stable, as indicated by the data in Table II. No significant change could be detected within 2 hr. of the time the reaction was carried out.

TABLE I

Effect of Cooling Time on the Scale Readings of DNS Method.

Sample	Time *min.*	Scale reading
1	1	71.5
2	2	71.5
3	3	71.8
4	4	72.0
5	5	72.2
6	6	72.0

TABLE II

Stability of the Color Developed in the DNS Method on Standing at Room Temperature

Sample	Time *min.*	Scale reading
1	1	72.5
2	15	73.0
3	30	72.5
4	45	73.0
5	60	73.2
6	120	73.0

TABLE III

Effect of Dilution of the Enzyme Sample on the Reproducibility of the DNS Method for Determining Amylase Activity

Sample	Enzyme	Scale readings	Units/ml.	Units/mg. enzyme ×100
	mg./ml.			
1	0.2	27.5	0.0069	3.45
2	0.2	27.6	0.0069	3.45
3	0.3	40.0	0.0104	3.47
4	0.3	40.0	0.0104	3.47
5	0.5	63.0	0.0178	3.55
6	0.5	63.0	0.0178	3.55
7	0.8	89.2	0.0280	3.50
8	0.8	89.2	0.0280	3.50
9	1.0	104.0	0.0351	3.51
10	1.0	104.0	0.0351	3.51

To determine the reproducibility of this method various concentrations of the same enzyme preparation were prepared and the results from each test used to calculate the activity of the preparation/100 mg. of material. Table III gives the results of these determinations. The results indicate that there is close agreement between activities determined using various concentrations of the enzyme. The small variation found is insignificant due to the dilution effect which is inherent in this system.

A comparison was made between the units of amylase activity for various preparations as determined by the DNS method and by the Willstätter *et al.* method. The calculated activities based on these determinations are given in Table IV. These results indicate a close agreement between the two methods. The units of amylase activity in the DNS method have been calculated using the same equation as used in the Willstätter *et al.* method. This equation is as follows:

TABLE IV

Comparison between the Activities of Various Pancreatin Preparations as Determined by the DNS Method and the Willstätter et al. *Method*

Sample	Units of amylase/mg. enzyme preparation ×100	
	DNS method	Willstätter *et al.* method
1	0.2	0.2
2	7.8	7.8
3	10.9	10.8
4	9.3	9.5
5	9.4	9.4
6	9.1	9.1
7	9.2	9.0
	4.9	5.0
	10.2	10.2
	2.6	2.7
11	10.4	10.4
12	9.0	9.0
13	7.0	7.0
14	3.2	3.2
15	4.7	4.7

$$K = \frac{1}{\text{time}} \log \frac{\text{g. hydrolyzable starch}}{\text{g. hydrolyzable starch} - \text{g. starch hydrolyzed}}.$$

In this equation K is used as an expression of amylase activity and K value/mg. of enzyme is an expression of enzyme purity. This value is the monomolecular constant of the enzymatic hydrolysis of starch under the test conditions. It has been found that only 75% (0.1875 g.) of the 0.25 g. of starch present in the test will be hydrolyzed (1,2,6,13). Therefore, the monomolecular constant can be calculated from the following equation:

$$K = \frac{1}{10} \log \frac{0.1875}{0.1875 - \text{g. maltose formed}}.$$

As was pointed out in the Willstätter *et al.* method, this equation can only be used accurately for expressing amylase activity when a K value between 0.001 to 0.03 is obtained. Similarly, in the DNS method, readings on the colorimeter between 40 and 70 gave the most accurate results. Therefore, enzyme solutions of proper concentrations which would give these results were used in all the determinations.

SUMMARY

A colorimetric method has been proposed for rapid determination of the amylase activity of pancreatin and other related pharmaceutical products which is believed to be more satisfactory than previously described methods for the routine analysis of a large number of samples.

The method is a modification of the Willstätter *et al.* method in which the milligrams of maltose formed are calculated by use of the dinitrosalicylic acid reagent for reducing sugars.

The amylase activity is, therefore, expressed in terms of Willstätter units.

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Ultraviolet Spectra of Some Indole Derivatives, Including Tryptophan and Gramicidin

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INTRODUCTION

These studies were undertaken in connection with work in this laboratory on the preparation and properties of derivatives of gramicidin (1,2,3). It was anticipated that the absorption spectra might be helpful in interpreting the reactions. Although the spectra were not of direct use for this purpose, the accumulated data, which may be useful for analytical purposes, are offered herewith.

MATERIALS AND METHODS

In the studies to be reported, a Beckman quartz spectrophotometer, Model D. U., was used. Observations were made between 2200 and 3500 Å at 50 Å intervals except in the vicinity of maxima, minima, and inflection points, where 10Å intervals were used. L-Tryptophan, skatole, indole, and 1,3-dimethylindole were commercial products. Crystalline gramicidin was contributed by the Wallerstein Labs. The other compounds studied were synthesized by B. A. Brandon, H. Fraenkel-Conrat and H. S. Olcott of this laboratory. All compounds were dissolved in 95% ethanol and compared with a blank of the same solvent.

RESULTS

The observed absorption spectra are shown in Figs. 1 and 2. The spectra of tryptophan and indole derivatives of known molecular weight (Figs 1 and 2) are plotted as molecular extinction coefficients ($\epsilon_M = \frac{D}{cx}$; c = moles/l.; x = cm.) against wavelength in Å. Concentrations of gramicidin, which was recently shown to be a mixture (4), and methylol gramicidin (5), are calculated as percentages instead of moles; hence, the spectra of these substances are plotted as $E^{1\%}_{1\,\text{cm.}}$, against Å (Fig. 1). It is apparent from the curves that, with respect to position, the spectrum of acetyltryptophan in 95% ethanol is very similar to that of gramicidin in the same solvent. From Table I the extinction coefficients also are seen to be nearly the same for the two substances, if 6 tryptophan units are assumed to be present in an average molecular weight of 3000 (6,7,8).

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[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

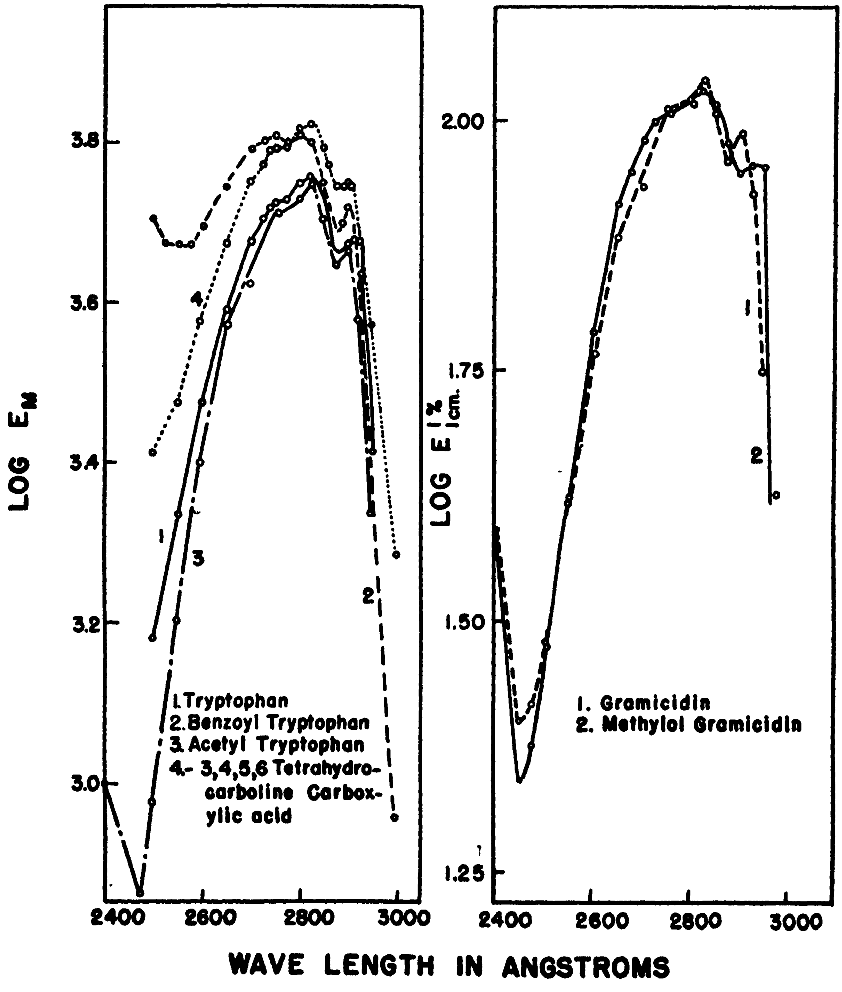

FIG. 1. Absorption spectra of tryptophan derivatives in 95% ethanol.

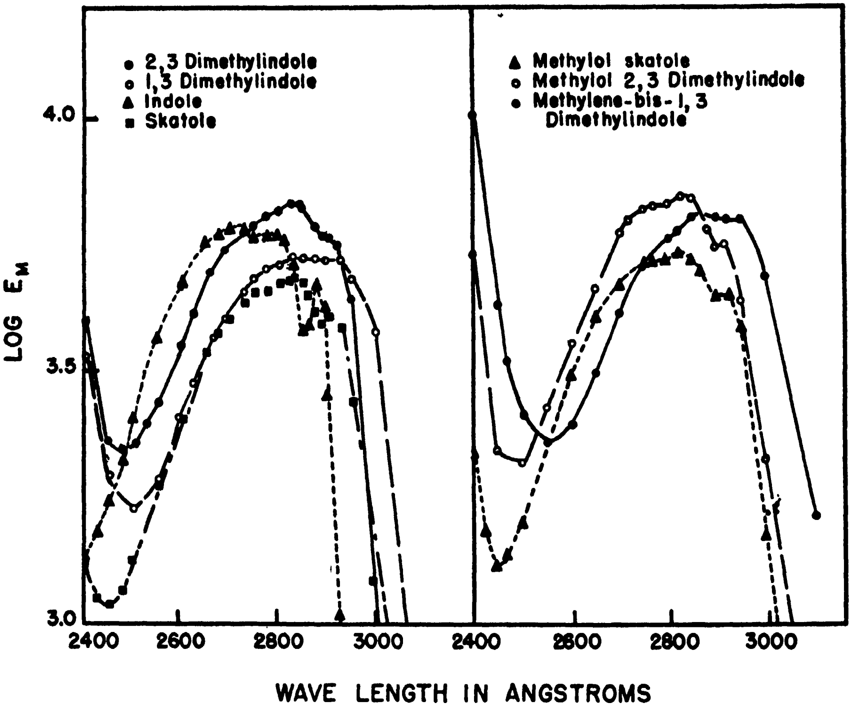

FIG. 2. Absorption spectra of indole derivatives in 95% ethanol.

For estimation of tryptophan content of the gramicidin samples no assumptions concerning homogeneity of gramicidin were made. The formula for any selected wave length is:

$$\text{Per cent tryptophan} = \frac{100 \times E^{1\%}_{1\,\text{cm.}}\ \text{gramicidin.}}{E^{1\%}_{1\,\text{cm.}}\ \text{acetyl tryptophan} \times \frac{247}{204}}$$

$E^{1\%}_{1\,\text{cm.}}$ of acetyl tryptophan = $\epsilon_M/24.7$, which from the table = 204, 227, and 157.5 at wavelengths 2740, 2825, and 2905, respectively. Use of the absorption of combined tryptophan, as in the acetyl derivative, rather than free tryptophan as the measure of tryptophan absorption, is justified practically by agreement of the results with chemical determinations, as well as logically. Densities at either of the two maxima or at the minimum could be used. Impurities are probably most likely to

TABLE I

Extinction Coefficients of Some Indole and Tryptophan Derivatives in Alcoholic Solutions

Compound	Band No. 1		Band No. 2		Band No. 3	
	Max Å	10^{-3} E_M	Max Å	10^{-3} E_M	Max Å	10^{-3} E_M
Solvent, 95% EtOH						
Indole	2725	6.12	2775	6.00	2875	4.85
Skatole	2750	4.55	2825	4.91	2900	4.12
2,3-Dimethylindole			2830	6.86	2900	5.98
1,3-Dimethylindole			2825,2920	5.44		
Methylol skatole	2750[a]	5.26	2830	5.48	2930	4.48
Methylol-2,3-dimethylindole			2825	7.05	2920	5.65
Methylene-bis (1,3-dimethylindole)			2870	6.59[b]	2950	6.39[b]
Tryptophan	2750	5.41	2800	5.77	2905	4.97
Acetyltryptophan	2740	5.09	2825	5.60	2905	4.89
Benzoyltryptophan	2750	6 42	2810	6.48	2900	5.24
3,4,5,6-Tetrahydro-4-carboline-5-COOH			2775	6.63	2885	5.61
Gramicidin[c]	2750	5.15[c]	2825	5.56[c]	2905	4.87[c]
Methylol gramicidin[c]	2750	5.13[c]	2825	5.42[c]	2950	4.39[c]
Solvent 25% EtOH						
Tryptophan	2700[a]	4.60	2810	5.30	2875	4.33
Acetyltryptophan	2650[a]		2800,2825	5.19	2905	4.81
Solvent H_2O						
Tryptophan	2710	4.60	2800,2825	5.19	2875	4.81

[a] Inflection.

[b] ϵ_M tryp.

[c] ϵ_M tryp, calculated by treating gramicidin like a single compound with mol. wt. 3000 and 6 tryp. per mol.

interfere at 2825 Å; however, errors in wavelength setting would be least deleterious on this comparatively broad band. We used the readings at 2825 and 2905 Å.

Thirty-eight samples of Wallerstein gramicidin of concentrations from 0.016 to 0.11 g/l. were analyzed by the above method. The percentage of tryptophan was found to be 39.3. The range of values was from 38 to 41%; the standard deviation was 1.12. By chemical analysis the figure, 39.6%, was obtained (3).

DISCUSSION

Any material in which tryptophan is the sole absorbing constituent can be analyzed by this procedure. In cases where the tryptophan content is known, solubilities can be determined by calculating tryptophan molarity on filtrates (3).

Direct spectrophotometric methods have been used (9,10) to calculate the tryptophan content of protein hydrolyzates. The tryptophan in unhydrolyzed gramicidin was tentatively estimated as 50% by McDonald (11), who, however, compared the ultraviolet spectrum of gramicidin in alcohol with that of pure tryptophan in water. This procedure, as the data of Table I show, would produce high results. The absorption spectra of some whole proteins in aqueous acid and alkaline solutions were obtained by Goodwin and Morton (9) and by Lerner and Barnum (12). The latter investigators used colorimetric tryptophan values to check the molecular absorption coefficients of the whole proteins. It is of interest that the tryptophan curve observed at pH 2 by these authors shows only one absorption band. The narrow band near 2900 did not appear, although readings were made at intervals of 25 Å or less.

The gramicidin curves of Hotchkiss and Dubos (13) and of Tishler, Stokes, Trenner and Conn (7) contain 3 sharply defined maxima at about 2700, 2820, and 2920 Å. The curve reported by McDonald, however, while containing a sharp maximum at 2910 Å, shows no trace of one below the principal band, 2815 Å. Our data, plotted as density vs. wavelength, show no definite maxima at a wavelength lower than 2820 Å.

It was of interest in connection with work on the reaction of formaldehyde with the indole groups (2) that introduction of methylol groups at the 1 position in the ring, as in methylol gramicidin, or of methylene groups at the 2 position as in methylene-*bis*-(1,3-dimethylindole), did not significantly alter the absorption spectra of the mother compounds. Similarly, introduction of a third ring formed by the crosslinking reaction of formaldehyde between the amino group of tryptophan and the carbon atom at the 2 position of the indole ring (13), produced absorption not remarkably different from that of tryptophan itself.

ACKNOWLEDGMENT

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SUMMARY AND CONCLUSIONS

Absorption spectra of alcoholic solutions of gramicidin, methylol gramicidin, tryptophan, acetyltryptophan, and 2,3,4,5-tetrahydro-4-carboline-5-carboxylic acid as well as of some indoles, methyl indoles, and methylol indoles are presented.

By the use of simple observations on the ultraviolet spectrum of unhydrolyzed gramicidin and on acetyltryptophan, the tryptophan content of gramicidin is estimated at 39.3%, which agrees with chemical values.

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The Biochemistry of Rubber Formation in the Guayule.
I. Rubber Formation in Seedlings [1,2]

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INTRODUCTION

The experiments to be reported in this series of papers were undertaken as one part of a general program to discover the chemical processes by which rubber is formed in plants. Strange as it may seem, essentially no work has been done on this subject in the past. Despite the continued and increasing importance of natural rubber production, rubber research has chiefly centered either on the conditions necessary for maximum rubber production or on the technology of the extracted rubber. A number of suggestions are to be found in the literature as to possible courses which rubber synthesis in the plant might conceivably take. These suggestions, which have been summarized in an earlier review (6), will be referred to below from time to time as appropriate. These few experiments on rubber formation which have been reported in the literature have, in the main, yielded results only in the sense that they have indicated paths which rubber synthesis does not take (for example see Stewart, Bonner and Hummer (13)). The thesis developed below, namely, that rubber formation proceeds from acetate as a starting material, has not heretofore been suggested, and does not, therefore, conflict with any experimental evidence heretofore adduced.

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[1] Report of work carried out as a cooperative project between the California Institute of Technology and the U. S. Department of Agriculture, Bureau of Plant Industry, Soils and Agricultural Engineering, Division of Rubber Plant Investigations.

[2] The authors wish to express their indebtedness to their colleagues who aided in various phases of this work: John O'Laughlin, Betty Jean Wood, Shigeru Honda and Charles Newman.

[3] Agent, U. S. Department of Agriculture, Bureau of Plant Industry, Soils and Agricultural Engineering, Division of Rubber Plant Investigations.

An understanding of the biochemistry of rubber formation is not only of intrinsic interest, but is of value also from the standpoint of application of this knowledge to the synthesis of natural rubber outside of the living plant. Given a knowledge of the rubber precursors, the enzymes, and the conditions of polymerization used by the plant, and ideas may be at least entertained as to the possible production, synthetically, of a natural rubber rather than of a rubber substitute.

GENERAL EXPERIMENTAL TECHNIQUE

The procedure used in these investigations consists in growing a rubber plant or plant part in synthetic nutrient solution. Controlled environmental conditions are used, so that endogenous rubber formation can be controlled with a considerable degree of exactitude. To the nutrient solution is added a compound or compounds regarded as being a possible rubber precursor or intermediate in rubber synthesis. If the compound causes an increase in the amount of rubber formed by the plant during a short experimental period, this compound is then seriously suspected of being an intermediate in rubber synthesis. The first two papers in this series summarize methods for, and results of, the screening of compounds for activity in supporting rubber formation. Final proof that the suspected compound is, in fact, an intermediate in rubber formation is based on experiments with isotopically tagged molecules of the intermediate, which will be related in the third paper.

The present work is restricted to the study of rubber production in the guayule, *Parthenium argentatum Gray*. Rubber is produced in this plant primarily in the parenchymatous cells of the pith and cortex of stems and roots, and may be accumulated to extraordinarily high concentrations as 20% or more of total dry weight of the plant. Knowledge concerning the environmental and cultural factors which condition rubber accumulation in the guayule are well understood and are summarized in Bonner and Galston (6).

Culture of Seedlings

The use of seedlings, rather than of mature plants, for these investigations, was originally dictated by the necessity of using small amounts of the various, and often difficultly obtainable, compounds to be tested as rubber precursors. The seedling plants were obtained from seeds of guayule strain 593, planted in washed river sand in flats and allowed to grow at a minimum greenhouse temperature of 70°F. until they attained a height of 2–3 cm. and an age of 2–4 months. They were then removed from the flat, washed free of sand, and transplanted individually to shell vials containing 15 cc. of a nutrient previously found (5) to support good growth of such seedlings. This nutrient is a standard nutrient solution of the composition shown in Table I. The shell vials were then placed in specially built boxes so that the entire root system of the plants was maintained in darkness. Each plant was held in place by a cork stopper in the mouth of the vial and each cork stopper was provided with a slit into which the plant fitted and a central hole through which the water lost by transpiration could be replaced. The substances to be tested for effect on rubber formation were added to the

TABLE I

Composition of Nutrient Solution Used for Cultivation of Seedling Guayule Plants

Salt	Conc.	Salt	Conc. of essential element
	mM/l.		*mg./l.*
$Ca(NO_3)_2$	1.20	H_3BO_3, B	0.55
$MgSO_4$	0.60	$MnSO_4$,Mn	0.56
		$ZnSO_4$, Zn	0.10
		Ferric tartrate, Fe	1.1
KNO_3	1.20	$CuSO_4$, Cu	0.23
KH_2PO_4	0.28	H_2MoO_4, Mo	0.08

nutrient solution. The nutrient solution and precursor, if any, were replaced by fresh solution once each week. The plants were grown in the air conditioned greenhouse under controlled temperature for 2–4 weeks in various experiments. Temperature control is an important factor since it has been shown earlier (4) that guayule plants grown under these conditions respond to low temperatures by greatly increased rubber concentration. Two temperature conditions were primarily used, *e.g.*, a constant temperature of 80°F., at which but little rubber accumulation normally occurs, and a

TABLE II

Reproducibility of Duplicate Lots of Guayule Seedlings with Respect to Growth and Rubber Content

Experiment M-35. Grown 80°F. day and night for 4 weeks. 40 plants per lot.

Lot	Dry wt./plant		Per cent total rubber	Total rubber/10 plants
	Roots and stems	Leaves		
	mg.	*mg.*		*mg.*
Initial	47	32	0.13	0.61
Final No. 1	70	81	0.14	0.98
2	76	77	0.13	0.99
3	82	78	0.12	0.98
4	77	72	0.14	1.08
5	77	69	0.08	0.62
6	75	75	0.15	1.12
7	69	76	0.14	0.97
8	70	76	0.12	0.84
9	76	83	0.14	1.06
10	79	79	0.16	1.26
Av.	75.1±1.34	76.5	0.13±0.0069	0.89±0.063

65°F. day temperature (8 hr.) combined with a 55°F. night temperature (16 hr.), a condition on the threshold of that required for rapid increase in rubber concentration.

Variability of Plant Material

In each experiment 40 plants were used for each condition or treatment tested. The variability to be expected in the plants used and harvested in lots of 40 plants each is shown in the experiments of Tables II and III, in which a series of replicate lots of 40 plants each were grown for 4 weeks either at 80°F., or at a 65°F. day, 55°F. night temperature. At the end of the experimental period, the plants were harvested, dried in a forced draft oven at 70°C., weighed, ground to 40 mesh in a Wiley mill and analyzed for rubber by the methods outlined below. The average deviation of the group mean of 40 from the overall mean, with respect to dry weight of stems and roots, was 4.4% at the higher temperature and 3.2% at the lower temperature. At the higher temperature the per cent of rubber contained in stems and roots did not change during the experimental period, while at the lower temperature it increased by over 50%. The per cent rubber in the plants maintained at the higher temperature varied on the average by 10.2% from the overall mean, while at the lower temperature an average deviation of 9.5% was found. The best estimate of the difference in rubber percentages needed for significance at the 5 and 1% levels, as between two lots of 40 plants each, respectively, are 0.062% and 0.084% at the higher temperature and 0.088% and 0.12% at the lower temperature. The nature of the experiments to be reported below precludes statistical evaluation of individual experiments since all of

TABLE III

Reproducibility of Duplicate Lots of Guayule Seedlings with Respect to Growth and Rubber Content

Experiment M-34: conducted parallel to experiment of Table II, but grown at 65°F. day and 55°F. night, for 4 weeks.

Lot	Dry wt./plant		Per cent total rubber	Total rubber/10 plants
	Roots and stems	Leaves		
	mg.	*mg.*		*mg.*
Initial	47	32	0.13	0.61
Final No. 1	61	53	0.22	1.34
2	58	55	0.25	1.45
3	59	54	0.22	1.30
4	66	52	0.18	1.19
5	63	61	0.27	1.70
6	63	56	0.19	1.20
7	63	56	0.19	1.20
Av.	62.0±1.20	56	0.21±0.013	1.38±0.068

the 40 plants of any one lot were required for rubber analysis. However, no result presented below will be taken as significant unless the differences exceed those required at the 1% level of significance as judged from Tables II and III. In addition, no experiment is reported unless it has been carried out and the effect confirmed in two to many experiments.

Methods of Rubber Analysis

The use of young plants has the advantage that such plants have a relatively low rubber content, 0–0.5%, and that proportionately large increases in rubber concentration can be affected in short experimental periods. This advantage of young tissues is offset by the fact that little information exists relative to the assay of rubber in such tissues. It was necessary, therefore, to determine conditions under which the rubber of young plants could be reproducibly determined using sample sizes of 100 mg. or less. In all cases, rubber was determined on the root-shoot portion of the plant after drying at 70°C. in a forced draft oven, and grinding to 40 mesh in a Wiley mill. The standard method of rubber determination in mature guayule is that developed by Spence and Caldwell (12), in which the resins are first extracted by acetone, the rubber then removed by benzene, and the rubber in the benzene extract estimated gravimetrically. A modification of this procedure consists in precipitating the rubber from the benzene extract with methyl alcohol and estimating the resultant precipitate turbidimetrically. The procedure used for this determination was as follows: a 100 mg. sample of the dry material was weighed into a 15 ml. centrifuge tube, and 10 cc. of acetone added. The material was allowed to extract with stirring for 2.5 hr. at 36°C., by which time complete extraction had occurred. The whole was then centrifuged, the acetone decanted, and the residue dried at 80°C. The residue was then extracted with 10 cc. of benzene for 1 hr. at room temperature by which time complete extraction had occurred. The material was again centrifuged and duplicate 2 cc. aliquots pipetted into 5 cc. of methyl alcohol. The turbidity was then determined in a Klett-Summerson colorimeter using a green filter to minimize the (small) absorption due to residual chlorophyll in the plant material. The results were standardized against the same procedure using either (*a*) varying amounts of purified rubber obtained by the Spence-Caldwell method, or (*b*) varying amounts of ground guayule plant of which the rubber content was determined using the Spence-Caldwell procedure. That the material obtained in the benzene extract of young plants is actually rubber was also shown by (*a*) concordance between the turbidimetric value and the value obtained by iodination of the extract using iodine monochloride, and (*b*) the preparation of the crystalline rubber tetrabromide from the benzene extract using the method of Willits, Swain and Ogg (15).

The rubber of rubber-bearing plants is known to contain a variety of molecular species of greatly differing molecular weights. In kok saghyz (Mashtakov,(8)) and in the guayule (3), young plants, and young parts of old plants, tend to contain more of their rubber in low molecular weight fractions than do older plants or plant parts. In the young guayule plants used in these studies, a portion of the rubber, possibly that of very low molecular weight, appears to be soluble in acetone. This results in the rubber values determined by the procedure above, which includes a preliminary acetone extraction, being too low. Rubber was, therefore, determined by an additional method carried out as above, but in which preliminary acetone extraction was omitted. A further complication is the fact that a wax contained in the rubber-bearing tissues, which is ordinarily largely removed by the preliminary acetone extraction, is also removed by benzene and precipitates out with methyl alcohol in the turbidimetric procedure. In the bromination method, however, the wax may be removed from the rubber tetrabromide, as the former precipitates slowly and may be removed by recrystallization of the rubber tetrabromide. Table IV gives a summary of determinations of apparent rubber on young guayule tissue by the turbidimetric and bromination procedures, with and without preliminary acetone extraction. These data show that acetone removes material from the low content shrub which is brominatable, precipitates with methyl alcohol, and behaves as rubber. The error introduced in the turbidimetric method by inclusion of the wax appears to be small. In most of the investigations below, rubber is reported as that determined without acetone extraction (total rubber), and that determined after preliminary acetone extraction, tentatively referred to as high molecular weight rubber. In certain critical experiments, rubber is additionally determined by the bromination method.

TABLE IV

Comparison of Various Methods of Rubber Analysis in Determination of Rubber Content of Young Guayule Plants

Method of rubber extraction	Per cent apparent rubber on dry weight basis	
	Turbidimetric	Bromination
Total rubber		
Initial benzene extract (no acetone ext.)	0.54	0.66
High molecular wt. rubber		
Benzene extract after acetone ext.	0.15	0.19

Experimental Results

Influence of Leaf Extracts on Formation of Rubber by Seedlings

The extracts of leaves of guayule plants which are actively making rubber appear to be able to support increased rubber formation in guayule seedlings. The leaf extracts which show this activity are made by extracting fresh leaves in a blendor with acetone. The acetone is then removed *in vacuo*, the residue taken up in H_2O, and this extract added to the nutrient given seedling plants. The results of a typical experiment with seedling plants are given in Table V. Extracts made from leaves of plants growing out of doors in the winter and shown by analysis to be actively increasing in rubber concentration caused as much as 97% increase in per cent total rubber in seedling plants under these conditions. Leaves from plants grown in a warm greenhouse and shown by analysis to be making little increase in rubber concentration were essentially devoid of this effect. Qualitatively similar results were obtained when leaf extracts were supplied to seedling plants grown under a 65°F. day, 55°F. night temperature regime. These results show that guayule plants may produce in their leaves a material which augments rubber formation in the stems and roots of guayule. Whether this

TABLE V

Influence of Acetone Extracts of Guayule Leaves on Rubber Formation in Seedling Guayule Plants, Experiment M-23

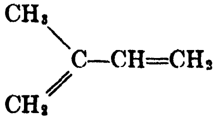
Test plants grown at 80°F. for one month.

Source of leaves used for ext.	Amt. of extract added mg. orig. lf./cc.	Dry weight of stems and roots/plant	Per cent total rubber	Rubber
		mg.		*mg./10 plants*
None (Final control)	None	61	0.37	2.26
Plants grown out doors, winter (Making much rubber)	2	58	0.73	4.24
	20	56	0.57	3.19
	200	54	0.67	3.62
Plants grown in greenhouse, 70°F. min. (Making little rubber)	2	60	0.45	2.70
	20	53	0.42	2.28
	200	56	0.43	2.41

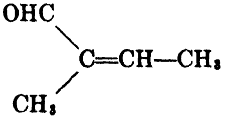
material is a precursor of rubber or is a factor which influences rubber formation in some other way cannot be stated from the present evidence. Experiments with defoliation (6) have shown, however, that, if the leaves are removed from a guayule plant rubber formation is greatly inhibited, even though the stem and root tissue contains abundant reserve carbohydrate. This evidence, together with that given in Table V, suggest that the leaves may make a precursor of rubber, a material other than carbohydrate, which may be transported to the stem and roots and there transformed to rubber. Identification of this naturally-occurring possible precursor will be of greatest interest, but further work on its isolation has as yet been unsuccessful. A more fruitful approach has been found to lie in the trial of known chemical compounds as possible rubber precursors.

Selection of Compounds for Trial as Precursors

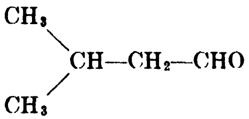
A comprehensive survey of the botanical and chemical literature (6) has presented several theoretical possibilities as to the mode of rubber formation in plants. This survey was used as a guide in the selection of materials for test. In general, rubber may be expected to be formed from a substance having the carbon skeleton of isoprene. Isoprene itself has not, however, been found in plants. Other compounds which have the same carbon skeleton and which do occur naturally are the following:



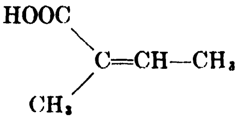
Isoprene



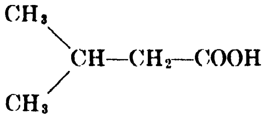
Tigaldehyde



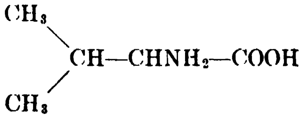
Isovaleraldehyde



Tiglic acid

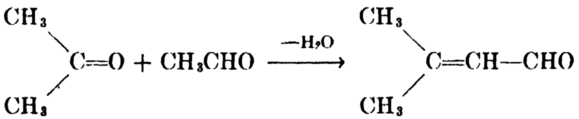


Isovaleric acid



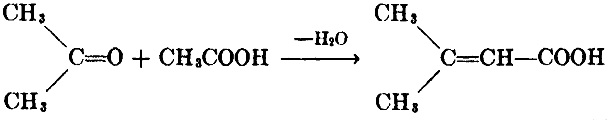
Valine

All of these substances have been tested for ability to support rubber formation in guayule. Prokofiev (10) and others have suggested that β-methylcrotonaldehyde may be the rubber precursor and that this substance may be formed from acetone and acetaldehyde as follows:



Acetone Acetaldehyde β-methyl-crotonaldehyde

β-Methylcrotonaldehyde has not been found in higher plants, but acetone has been isolated from guayule leaves (7) and acetaldehyde is of common occurrence, as is the dimer of β-methylcrotonaldehyde, citral. These substances were, therefore, tested for ability to support rubber formation. The guayule contains a variety of simple terpenes, mainly C_{10} compounds such as α-pinene, which, like rubber, are composed of isoprene units or their derivations. These leaf oils were, therefore, tested for ability to support rubber formation, even though the transformation of such cylic terpenes to rubber is most unlikely. Finally, the following reaction was considered in relation to rubber formation:



Acetone Acetic acid β-Methylcrotonic acid

This reaction is the mechanism of rubber formation in guayule suggested by the experiments reported below.

Rubber Formation as Influenced by Various Added Substrates

Seedling plants were grown as detailed above, and the various substances to be tested for effect on rubber formation added to the nutrient medium. Table VI gives a summary of the substances tested and found to be ineffective. Added carbohydrate, in the form of pyruvate or sucrose, is without effect on rubber production. All of the

5 carbon compounds with the carbon skeleton of isoprene were ineffective, as was citral. Acetaldehyde is without influence on rubber production, as are also the common plant acids citric and fumaric. The conclusion to be drawn from Table VI is, then, that none of the naturally-occurring compounds having an obvious structural relationship to rubber are active in augmenting rubber formation under the conditions tried.

TABLE VI

Summary of Experiments on Compounds Tested and Found Ineffective in Influencing Rubber Formation in Seedling Guayule Plants

All substances adjusted to pH 5.5.

Expt.	Temp. under which grown	Length exptl. period	Substance added	Conc. range tested	Effect on growth in dry wt.	Rubber content
	°F.	*weeks*				
M-8	80	2	Pyruvic acid	10-1000 mg./l.	100 mg./l. inhibitory	None
			Sucrose	5-20 g./l.	Reduces growth	None
M-8-A	80	2	Crotonaldehyde	1-100 mg./l.	1 mg/l. toxic	None
			Citral	10 mg./l.	None	None
			Acetaldhyde	10-100 mg./l.	Increases growth	None
M-9	80	2	Isovaleraldehyde	10-100 mg./l.	None	None
M-14	80	2	α-Pinene	10-90 mg./l.	None	None
M-15	80	2	Limonene	10-90 mg /l.	None	None
			Guayule leaf oil	1-9 mg./l.	None	None
M-32	80	4	Succinic acid	100 mg./l.	Toxic	None
			Fumaric acid	100 mg./l.	Reduces growth	None
			Citric acid	100 mg./l.	Reduces growth	None
			Isovaleric acid	100 mg./l.	Reduces growth	None
M-42	65 day 55 nt.	4	Tigaldehyde	10-100 mg./l.	No effect	None
			Tiglic acid	10-100 mg./l.	No effect	Reduces
			Valine	10-100 mg./l.	No effect	No effect
			Leucine	10-100 mg./l.	No effect	No effect

Acetate and Rubber Formation

The addition of acetate to the nutrient in which guayule seedlings are grown causes an increase in the amount of rubber accumulated as is shown in Table VII. In this experiment, seedlings of the same lot were grown with various concentrations of acetate, both at high temperature (80°F. day and night) and at lower temperature 80°F. day and 50°F. night). Regardless of the temperature in which the seedlings were grown, percentage of rubber was doubled by addition of acetate to the medium at a concentration of 100 mg. NaAc per liter. The greatest accumulation occurred at the

TABLE VII

Effect of Acetate on the Formation of Rubber by Guayule Seedlings

Expt. M-5. Grown at temperature indicated for 4 weeks. Acetate given as Na acetate, pH 5.5. Initial rubber per cent 0.00.

Addenda to nutrient solution	Temp. for growth	Dry wt./plant		Per cent total rubber	Rubber
		Stem and roots	Leaves		
	°F.	mg.	mg.		mg./10 plants
None, final control plants	80 cont.	42	69	0.14	0.59
Acetate 10 mg./l.	80 cont.	41	64	0.25	1.02
Acetate 100 mg./l.	80 cont.	33	50	0.27	0.89
Acetate 1000 mg./l.	80 cont.	9	13	0.00	0.00
None, final control plants	80 day 50 night	16	19	0.31	0.50
Acetate 10 mg./l.	80 day 50 night	16	19	0.44	0.70
Acetate 100 mg./l.	80 day 50 night	13	13	0.57	0.74
Acetate 1000 mg./l.	80 day 50 night	7.6	9.6	0.28	0.21

higher temperature although the greatest effect on rubber percentage was at the lower temperature. A definite effect of acetate on rubber accumulation was found with concentrations as low as 10 mg./l. a concentration which was without any considerable effect on growth. One thousand mg. of acetate/l., which was definitely toxic to the plants, was ineffective in augmenting rubber accumulation. The effect of acetate on

TABLE VIII

Inhibition of the Rubber Formation Effect of Acetate by Fluoroacetate

Plants out of doors. Experiment M-30. Plants grown 4 weeks.

Treatment	Wt. per plant		Rubber dry wt		Total rubber/10 plants
	Stems and roots	Leaves	Total	High mol. wt.	
	mg.	mg.	per cent	per cent	mg.
Initial control	48	49	0.58	0.00	2.78
Final control	61	47	0.51	0.00	3.11
Na acetate 100 mg./l.	81	63	0.74	0.09	6.00
Same+NaFAc 10 mg./l.	95	75	0.49	0.09	4.65
Same+NaFAc 30 mg./l.	53	45	0.46	0.09	2.44
Same+NaFAc 60 mg./l.	50	46	0.37	0.09	1.85

rubber accumulation has been qualitatively confirmed in 7 further experiments with seedlings, the increases in rubber concentration ranging from 25% to over 3-fold in separate experiments. The variations in effectiveness are undoubtedly due to variations in the initial status of the seedlings. In contrast to the effects of acetate, are those of fluoroacetate, which is an inhibitor of acetate metabolism in microbial systems (2). Table VIII shows that fluoroacetate inhibits the effect of acetate in increasing rubber accumulation. In this experiment, seedling plants were grown in the standard manner and the supplying of acetate brought about a doubling of total rubber content over that found in the non-acetate controls. The further addition of fluoroacetate in a concentration of 10 mg./l., while it increased growth somewhat, decreased rubber accumulation. A 3 times higher concentration of fluoroacetate, which decreased growth appreciably, abolished the effect of acetate on rubber accumulation altogether. While the effects of fluoroacetate are not yet completely clear, particularly as to the growth-promotive effects which this substance seems to exert at low concentrations, still it seems justifiable to conclude that this substance inhibits the effect of acetate in increasing rubber accumulation.

TABLE IX

Effect of Acetate on Rubber Formation in Defoliated Guayule Seedlings

Plants incubated in greenhouse for 4 weeks.

Expt. no	Addenda	Temp condition	Dry wt. of stems and roots	Per cent total rubber	Rubber
		°*F.*	*mg./plant*		*mg./10 plants*
M-21	None	65° day, 55° nt.	44	0.15	0.66
	Acetate 100 mg./l.	65° day, 55° nt.	41	0.47	1.93
M-24	None	80° day and nt.	58	0.33	1.91
	Acetate 100 mg./l.	80° day and nt.	46	0.31	1.43

It has been shown above that leaf extracts promote rubber formation in seedling guayule plants, and that acetate possesses a similar effect. Defoliated plants, on the other hand, produce, in general, little or no rubber. It is logical to inquire, therefore, whether acetate can replace leaves in bringing about rubber formation in defoliated plants. In the experiments of Table IX, the seedling plants were completely defoliated at the time they were transplanted to vials and were maintained in a defoliated condition. The data of Table IX show that addition of acetate resulted in an increase in rubber in the defoliated plants kept at low temperature, but that no effect was obtained with acetate given to plants in a separate experiment and maintained at high temperature.

Effect of Acetone on Rubber Formation

Acetone, like acetate, exerts an effect on rubber formation by guayule seedlings, as is shown in the experiment of Table X, where the addition of acetone resulted in as large or larger increases in rubber concentration as did the addition of acetate. The effects of acetone and acetate do not appear to be additive in any way and, if anything,

TABLE X

Effect of Acetone and Acetate and Acetone in Combination on Rubber Formation in Guayule Seedlings

Grown at 65°F. day, 55°F. night for two weeks.

Addenda	Dry wt./plant		Per cent total rubber	Rubber/10 plants
	Stems and roots	Leaves		
				mg.
Initial plants	27	115	0.20	0.54
None (final control)	71	146	0.30	2.13
Acetone 100 mg./l.	70	145	0.44	3.08
Acetone 1000 mg./l.	65	176	0.52	3.38
NaAc 10 mg./l.	65	129	0.52	3.38
NaAc 100 mg./l.	60	111	0.40	2.40
Acetone 100 mg./l.+NaOAc 10 mg./l.	43	141	0.36	1.55
Acetone 100 mg./l.+NaOAc 100 mg./l.	50	142	0.36	1.80
Acetone 1000 mg./l.+NaOAc 10 mg./l.	64	126	0.40	2.56
Acetone 1000 mg./l.+NaOAc 100 mg./l.	48	127	0.39	1.87

the two substances may interfere with one another in their effects on rubber formation. In addition, considerable depression in growth resulted from use of acetone and acetate together, with a resultant depression in total amount of rubber formed per plant.

Effect of Glycerol on Rubber Formation

A further substance, glycerol, was found to influence rubber accumulation as do acetate and acetone. Glycerol is relatively non-toxic in concentrations as high as 1-3 g./l., and addition of glycerol in these concentrations brought about substantial increases, both in rubber concentration and in total amount of rubber as is shown in Table XI. Addition of acetate in the presence of glycerol did not further increase the accumulation of rubber.

Effect of Acetate and Acetone on Older Plants

It was desired to test the effect of acetate and of acetone on older plants as well as on seedlings. To this end, use was made of a group of plants 8 months old and growing in gravel culture in two quart containers. The plants were divided into 13 lots of 20 plants each and one lot harvested immediately (September 25) as an initial control. The further 12 lots were equally divided between a greenhouse maintained with a minimum of 70°F. and an out of doors plot. Each group of 6 lots of 20 plants was randomized and 6 treatments applied, consisting of nutrient solution or nutrient plus the addenda shown in Table XII. The nutrients were supplied daily until December 1 (approximately 2 months), at which time the plants were harvested. Each group

TABLE XI

Effect of Glycerol on Rubber Formation in Seedling Guayule Plants

Plants grown at 65°F. day, 53°F. night for 4 weeks.

Addenda	Dry wt./plant		Per cent total rubber	Rubber/10 plants
	Stems and roots	Leaves		
	mg.	*mg.*		*mg.*
None	65	82	0.20	1.30
Glycerol 1 gm./l.	55	78	0.55	3.02
Glycerol 3 gm./l.	50	90	0.73	3.65
Na acetate 100 mg./l.	61	91	0.67	4.08
Glycerol 1 g./l.+acetate 100 mg./l.	64	84	0.37	2.37
Glycerol 3 g./l.+acetate 100 mg./l.	59	75	0.62	3.66

TABLE XII

Lack of Effect of Acetate and/or Acetone on Rubber Formation in 8 Month Old Guayule Plants

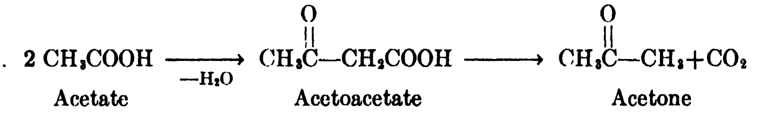
Experiment started September 25: harvested December 1.

Treatment	Dry wt. g./plant		Per cent rubber	Rubber/plant
	Stems and roots	Leaves		
				mgs.
Initial control	2.3	4.6	1.43	33
Green house grown				
Final Control	5.0	3.6	1.76	88
Acetate 100 mg./l.	5.2	3.0	1.80	94
Acetate 1 g./l.	5.5	3.2	1.60	88
Acetone 1 g./l.	4.5	2.8	1.24	56
Acetone 10 g./l.	4.6	2.4	1.40	64.5
Acetate 100 mg./l. and acetone 1 g./l.	4.7	3.2	1.43	67
Outdoors grown				
Final control	7.1	4.4	2.72	193
Acetate 100 mg./l.	7.0	4.4	2.50	175
Acetate 1 g./l.	9.0	5.5	2.66	239
Acetone 1 g./l.	6.8	4.0	2.96	201
Acetone 10 g./l.	6.8	3.6	2.82	192
Acetate 100 mg./l. and acetone 1 g./l.	7.1	4.0	2.26	160

was harvested as 10 lots of 2 plants each. The plants were dried, weighed, and analyzed for rubber by the turbidimetric method after acetone extraction. Table XII shows that, in no case, was any striking effect obtained as the result of treatment with acetate and/or acetone, and statistical treatment of the data has, therefore, been omitted. Apparently the older plant is not limited by acetate and/or acetone to the extent that the seedling is so limited.

Intermediary Metabolism of Acetone and Acetate

It has been shown that acetate and acetone influence the production of rubber by guayule seedlings. The two substances appear to be interchangeable rather than additive in their effects. It is known from work on other organisms, such as *Clostridium* (14), that acetone is made from acetate through the intermediary of acetoacetate according to the following reaction:



$$.\ \underset{\text{Acetate}}{2\,CH_3COOH} \xrightarrow[-H_2O]{} \underset{\text{Acetoacetate}}{CH_3\overset{\overset{O}{\|}}{C}-CH_2COOH} \longrightarrow \underset{\text{Acetone}}{CH_3\overset{\overset{O}{\|}}{C}-CH_3+CO_2}$$

It is of interest to discover, therefore, whether a similar mechanism is operative in guayule. The data of Table XIII show that acetoacetate is active, as are acetate and acetone, in supporting rubber formation in seedling guayule plants. This would be expected on the basis of the above mode of acetone formation. Proof that the mechanism is operative in guayule must rest on other types of experiments such as those involving isotopic carbon.

TABLE XIII

Effectiveness of Acetoacetate and β-Methylcrotonic Acid in Supporting Rubber Formation in Seedling Guayule Plants

Experiment M-44. Grown at 65°F. day, 55°F. night for 4 weeks. All solutions adjusted to pH 5.5. Rubber determined by bromination method.

Addenda to nutrient solution	Dry wt./plant		Per cent total rubber	Rubber/10 plants
	Stems and roots	Leaves		
	mg.	*mg.*		*mg.*
Initial control plants	6.5	24	—	—
Final control plants	39	56	0.12	0.47
Acetoacetate, 10 mg./l.	43	65	0.33	1.42
β-Methylcrotonic acid, 10 mg./l.	38	66	0.23	0.87

The evidence presented up to the present point does not permit of a decision as to whether acetate is incorporated into rubber *per se* or is involved only as a precursor of acetone. Evidence that both possibilities are actually realized is the fact, shown in Table XIII, that β-methylcrotonic acid[4] is active in supporting rubber formation. This compound may be regarded as a condensation product of one molecule each of acetate and acetone, a reaction involving the loss of one molecule of water, and possesses the branched chain structure characteristic of isoprene.

Discussion

It has been shown above that, of all the various substances related to the carbon skeleton of isoprene, which were investigated as to possible effects on rubber formation in guayule seedlings, only acetate, and substances metabolically related to acetate, were effective in promoting rubber formation. Thus glycerol, effective in promoting rubber formation, is a preferred substrate for the production of acetate and its derivatives by *Clostridium* species. Acetoacetate and acetone are similarly derivatives of acetate both in *Clostridium* and in higher animals. β-Methylcrotonic acid, the final substance here found effective in rubber formation is not yet known as a metabolic product in any organism, but it does bear a close relation both to acetate and acetone. It is a striking fact that the seedling plants used in this investigation were not able to utilize either sucrose or pyruvate as substrates for increased rubber formation, although acetate must ultimately derive from sucrose through pyruvate. This would apparently signify that guayule seedlings are limited in their rubber formation by their capacity to convert pyruvate to acetate, particularly since their utilization of acetate indicates that the later steps are not limiting.

The fact that leaf extracts contain a material capable of supporting increased rubber synthesis in guayule seedlings raises the question of whether the active principle of leaves is acetate or one of its derivatives. This question cannot yet be answered; it is known only that the active principle is soluble in acetone.

Investigation of the mechanism of fatty acid synthesis in microorganisms (1,16) and in higher animals (11) has shown that this synthesis proceeds stepwise through the condensation of acetate or a derivative of acetate with acetoacetate, followed by reduction of this product and condensation with a further acetate, *etc.* The present data suggest a far reaching analogy between fatty acid formation and rubber formation, with β-methylcrotonic acid possibly replacing acetate as the fundamental repeating unit. Complete elucidation of this possible parallel which can be dealt with only in a long range extension of this work.

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[4] The authors are indebted to Dr. A. J. Haagen-Smit and Mr. Carl Redeman for the supply of β-methylcrotonic acid.

SUMMARY

1. Investigation of the mode of formation of rubber in plants was carried out with the aid of nutrient solution-grown seedlings of the guayule. Substances to be tested for effect on rubber formation were added to the nutrient in which the plants were grown.

2. Extracts of leaves of guayule plants actively engaged in rubber formation when added to the nutrient solution of guayule seedlings bring about increased rubber formation. Leaves of plants not engaged in active rubber formation are without such effect.

3. Glycerol, acetate, acetoacetate, acetone, and β-methylcrotonic acid were the only organic substances found to increase rubber formation in guayule seedlings.

4. On the basis of the compounds found to increase rubber formation it is suggested that the reactions involved may approximate:

$$\begin{tikzcd}
\text{acetate} \arrow[r] & \text{acetoacetate} \arrow[r] & \text{acetone} & \\
 & & + \arrow[r] & \beta\text{-methylcrotonic acid.} \arrow[dd] \\
 & & \text{acetate} & \\
 & & & \text{rubber}
\end{tikzcd}$$

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Chetomin[1] an Antibiotic Substance from *Chaetomium cochliodes*. III. Composition and Functional Groups[2,3]

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Chetomin is an antibiotic substance produced by a strain of *Chaetomium cochliodes* (1,2), and by *Chaetomium spirale*, and by *Verticillium cinnabarinum* (= *Acrostalagmus cinnabarinus*) (3). The substance is secreted by the *Chaetomium* into the culture medium, but is present in larger quantities in the mycelium. Chetomin is active against gram-positive bacteria such as *Staphylococcus aureus* at dilutions as high as 1:500,000,000. Although the substance has not been obtained in crystalline form, improved methods of purification now yield a white powder of constant melting point (215°C.) and composition, so the substance seems to be pure enough to justify chemical investigation. The lack of crystallinity of chetomin and its principal derivatives, and the consequent difficulties of purification cause certain analytical data to deviate somewhat from the calculated values.

The purified substance has now been found to have a composition agreeing best with the formula $C_{16}H_{17}O_4N_3S_2$. Determinations of the molecular weight favor the simple formula rather than a higher multiple.

A preliminary survey has been made of the effect upon the activity of chetomin of a number of reagents expected to give information concerning functional groups likely to be present. Such tests, as Brown and du Vigneaud (4) have pointed out, make it possible to obtain preliminary information with a minimum expenditure of material. The substance was found to be readily inactivated by oxidizing agents, alkali, and hot acids, but other reagents were generally without effect (Table I).

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[1] Spelling changed from *Chaetomin* at the suggestion of E. J. Crane, Editor of *Chemical Abstracts.*

[2] Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

[3] Supported in part by a grant from the Commonwealth Fund of New York.

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TABLE I

Effect of Chemical Reagents on the Bacteriostatic Activity of Chetomin

Reagent	Conditions	Inactivation observed
Aeration	24 hr., 25°C.	−
Iodine	0.05 *N*, 24 hr., 25°C.	+
Bromine	0.5 *N* 24 hr., 25°C.	+
Hydrogen peroxide	2.5%, 24 hr., 25°C.	+
Chloramine T	0.5%, 24 hr., 25°C.	+
Hydrogen-PtO	1 hr., 25°C.	−
Hydrogen-Pd	1 hr., 25°C.	−
Acetic anhydride	24 hr., 25°C., pyridine as solvent	−
Benzyl chloride	24 hr., 25°C., pyridine as solvent	−
p-Bromophenylisocyanate	0.3%, 24 hr., 25°C., dioxane as solvent	−
Diazomethane	2 hr., 25°C., ether as solvent	−
Methyl iodide	pH 8.0, 24 hr., 25°C.	−
Formaldehyde	20%, 100°C., 0.5 hr.	−
Ninhydrin	0.03%, pH 5, 24 hr., 25°C.	−
Nitrous acid	7% in 30% AcOH, 24 hr., 25°C.	−
Hydroxylamine	0.25% in 50% pyridine, 24 hr., 25°C.	−
Sodium bisulfite	0.03 *M*, pH 5, 24 hr., 25°C.	−
2,4-Dinitrophenylhydrazine	Brady's reagent	−
Hydrochloric acid	20%, 120°C.	+
Alkali	Borate buffer, pH 9.0, 24 hr., 25°C.	+

Zerewitinoff determination showed the presence of 4 active hydrogen atoms. Quantitative acetylation by Stodola's (5) method indicated the presence of one group that could be acetylated. Methoxyl was absent, but one methylimide group was present. No ketonic or aldehydic group could be detected. No hydrogen was absorbed in the presence of platinum or palladium catalysts.

There was no evidence of the presence of either free acidic or basic groups. The substance could not be removed from solution in organic solvents by washing with aqueous acid or alkali and was not precipitated by picric acid or mercuric chloride. Its antibacterial properties were not affected by diazomethane, and no methoxyl was introduced by this reagent. On saponification, about 3 equivalents of alkali were absorbed per mole.

Chetomin reacted with sodium plumbite with the formation of lead sulfide. Desulfurization of chetomin with Raney nickel (6) led to a product with the composition $C_{16}H_{19}O_3N_3$. The same product was obtained by reduction with zinc dust and acetic acid. The reactions have led to a loss of 2 sulfur and 1 oxygen atoms, and an uptake of only two hydrogen atoms. The results suggest the presence of a linkage involving 2 sulfur and one oxygen atoms, such as a thiosulfenic ester grouping. The product is termed *desthiochetomin.*

No amino nitrogen could be detected in chetomin or desthiochetomin by the Warburg and Christian (8) modification of the van Slyke method. Since ammonia was liberated on hydrolysis of chetomin with either strong acid or alkali, and carbon dioxide was formed during alkaline hydrolysis, the presence of a primary amide group seemed possible. The reaction of xanthydrol with chetomin to yield a compound containing one xanthydryl group, and with desthiochetomin to yield a compound containing two xanthydryl groups, tended to confirm the presence of a primary amide grouping. The indole nucleus is known to react with xanthydrol (8).

Desthiochetomin gave positive tests for the presence of an indole nucleus with glyoxylic acid and with *p*-dimethylaminobenzaldehyde. Distillation of desthiochetomin with zinc dust yielded an unidentified indole derivative, apparently a lower alkyl homolog. Color reactions suggest that the imino group of the indole is unsubstituted. The ultraviolet absorption spectrum also indicated that an indole nucleus was present in chetomin.

A resemblance between chetomin and ergine, derived from a product of another Ascomycete, *Claviceps purpurea*, seems worth noting, since ergine has the composition $C_{16}H_{17}ON_3$, and includes an indole nucleus, a methylimide, and a primary amide grouping (9).

EXPERIMENTAL

Purification. Chetomin (1.0 g.), obtained and partly purified as described (2), was dissolved in 200 ml. of benzene and poured on a 5.0 cm. adsorption column of Darco G-60. The column was washed with 200 ml. of benzene, and then eluted with benzene containing 1.0% of anhydrous methanol. The benzene-methanol eluate was evaporated to dryness. The residue was taken up in 25 ml. of chloroform, and added to 250 ml. of ice cold petroleum ether. The precipitate was filtered off and dried *in vacuo.* The substance formed a white powder (0.850 g.), and its solutions were only slightly colored. The purified substance sintered at 185°C. and melted at 215°C. with the formation of gas. Chetomin was strongly dextrorotatory: with a 1% solution in chloroform $[\alpha]_D^{22}$ was + 360°.

Composition. A portion of the substance was separated into two fractions by repeated partial precipitation from acetone with ether. The two fractions showed no detectible difference in melting point or antibacterial potency.

Anal. Calcd. for $C_{16}H_{17}O_4N_3S_2$: C, 50.66; H, 4.48; N, 11.08; S, 16.89. Found: less soluble fraction: C, 51.36; H, 4.23; N, 11.10; S, 16.77; more soluble fraction: C, 50.70; H, 4.38; N, 10.80; S, 17.44.[5, 6]

The substance decomposed at the melting point of camphor, and was too insoluble in camphene, cyclohexanol, and carbon tetrabromide for cryoscopic molecular weight determination. Determinations in ethylene bromide yielded a value of 310. Determinations run by the Signer method (10) with acetone as solvent and azobenzene as standard reached equilibrium very slowly and gave a value of 670, which suggests association in this solvent. Determination of groups capable of acetylation by Stodola's method (5) gave 4.35% (as OH); calc. for one hydroxyl group, 4.50%. A determination of methylimide gave 6.17% (as CH_3); the calculated value for one methylimide group is 3.95%.

Inactivation Experiments. The chetomin used in these experiments was generally an emulsion in 5% aqueous gum-acacia solution, and contained 1.0 mg. of chetomin/ml. with an activity of 300,000 dilution units/ml. against *Staph. aureus.* For those reagents requiring anhydrous conditions, dry powdered chetomin was used. The bacteriostatic activity was determined by the agar streak method (11) using 4 organisms: *Staph. aureus, Bacillus subtilis, Bacillus mycoides, and Sarcina lutea.* In all cases, the results with one organism were paralleled by those with the others. The possibility that the reagents might have an inhibiting effect on the test organisms was checked by experiments in which chetomin was omitted. Where necessary, the reagent was removed before the final testing. For example, in the test with iodine, 1.0 ml. of the chetomin emulsion was mixed with 1.0 ml. of 0.1 *N* iodine and kept at 25°C. for 24 hr. Then 1.0 ml. of 0.1 *N* $Na_2S_2O_3$ was added, the mixture diluted with 7.0 ml. of alcohol, and tested. The final mixture was found to have an activity of 1000 dilution units/ml. against *S. aureus.* A control experiment with iodine omitted gave 30,000 units/ml., and another with chetomin omitted, less than 1000 units.

Alkaline Hydrolysis. (a) Saponification Equivalent. Samples (10–15 mg.) of chetomin were refluxed in 5.0 ml. 0.1 *N* KOH (with CO_2 excluded) and the mixture was then titrated with 0.02 *N* HCl. Hudrolysis for 2 hr. gave an equivalent weight of 137; for 4 hr., 123; and for 24 hr., 115.

Alkaline Hydrolysis. (b) Formation of CO_2. Samples (approx. 10 mg.) were hydrolyzed in sealed tubes for 16 hr. at 140°C. with 1 ml. of 1.0 *N* KOH. The contents were transferred to a small flask, acidified with 2 ml. 1.0 *N* HCl and refluxed in a stream of CO_2-free nitrogen (5 cc./min.) for 1.5 hr. The gases from the top of the condenser were passed through a washer containing concentrated H_2SO_4, through a tube containing Dehydrite, then through a weighed tube of Ascarite. Found: CO_2, 13.2%. Calc. for $C_{16}H_{17}O_4N_3S_2$: 11.7%.

Alkaline Hydrolysis. (c) Formation of a Volatile base. Chetomin (10 mg.) was hydrolyzed in a sealed tube for 24 hr. at 140°C. with 2.0 ml. of 1.0 *N* KOH. The tube was then chilled and opened, and its contents transferred to an aeration appartus. By aeration into boric acid and titration with acid, it was found that 4.5% of volatile

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[5] A sample of a substance believed identical, kindly sent by Prof. Ernst Gäumann, Eidg. Technische Hochschule, Zurich, contained C, 49.07; H, 4.61; N, 11.17; S, 17.64.

[6] Microelementary and certain functional group analyses were performed by Joseph F. Alicino, Metuchen, N. J.

nitrogen had been formed. The residue after aeration was acidified with 2 ml. conc. H_2SO_4, evaporated, and submitted to a micro Kjeldahl determination. Found, 6.0% of (nonvolatile) nitrogen.

Chetomin (100 mg.) was hydrolyzed for 24 hr. at 140°C. with 5.0 ml. of 1.0 *N* KOH in a sealed tube containing in a separate compartment 5.0 ml. of 2.0 *N* H_2SO_4. The tube was cooled, the contents mixed with the acid, and opened. The contents were introduced into a Parnas-Wagner Kjeldahl distillation apparatus and steam-distilled into a solution of 95 mg. picric acid in 3.0 ml. of alcohol. The picrate, crystallized from aqueous alcohol, formed yellow needles melting over a wide range (158–165°C.). Melting point data for ammonium picrate seem to be lacking from the literature, probably because the compound loses ammonia on heating (12). Anal. Calcd. for $C_6H_6N_4O_7$ (ammonium picrate): C, 29.27; H, 2.44; N, 22.76. Found: C, 30.42; H, 2.34; N, 20.82.

Acid Hydrolysis. Ammonia Formation. Chetomin (20 mg.) was hydrolyzed for 22 hr. with 10.0 ml. of 6.0 *N* HCl in 50% alcohol. The hydrolyzate was transferred to a Parnas-Wagner apparatus and the ammonia distilled as usual. The volatile nitrogen amounted to 3.42%, while the calculated value for one equivalent is 3.43%.

Reaction with Xanthydrol. Chetomin (50 mg.) was dissolved in 2.0 ml. of glacial acetic acid and 1.5 ml. of a 4.6% alcoholic solution of xanthydrol was added. The mixture was kept at 60°C. for 2 hr. and 2.5 ml. of water was added and the precipitate centrifuged off. The product was reprecipitated from acetic acid by adding water, washed with water until free of acid, and dried. The dry product was a bluish solid which weighed 59 mg., sintered at 120–130°C. and melted at 139°C. Anal. C, 61.21; H, 3.77; N, 5.90. The results suggest that the product consists largely of a dixanthydryl derivative. The product was not bacteriostatic.

Desthiochetomin (a). Chetomin (200 mg.) was dissolved in 100 ml. of 95% ethyl alcohol, and 5.0 ml. of water and approximately 5 g. of Raney catalyst (6) were added. The mixture was refluxed 5 hr., and the catalyst then centrifuged off and washed with ethyl and methyl alcohol. The supernatant solutions were evaporated to dryness *in vacuo*, and the residue was dissolved in 20 ml. of chloroform and added to 100 ml. of petroleum ether. The precipitate was centrifuged off, washed with petroleum ether and dried *in vacuo*. The product (150 mg.) was a white powder which sintered at 145–150°C. and melted at 180°C. The product had no detectible antibacterial properties. Anal. Calc. for $C_{16}H_{19}O_3N_3$: C, 63.78; H, 6.31; N, 13.95. Found: C, 62.85; H, 6.25; N, 13.75. Desthiochetomin was dextrorotatory; with a 1% solution in chloroform, $(\alpha)^{22}_{D}$ was + 202. The molecular weight, according to the Signer method was 547, which suggests association. The compound contained 3.8% of methylimide (calc. as CH_3) and 4.05% of acetytable groups (5) (calc. as OH); the theoretical values for single groups are 5.0% and 5.6%, respectively.

Desthiochetomin (b). Chetomin (100 mg.) was refluxed for 2 hr. in 5.0 ml. of glacial acetic acid with 1.0 g. of zinc dust. The mixture was then filtered, evaporated to dryness, and the residue dissolved in 5.0 ml. of chloroform. The chloroform solution was added to 40 ml. of petroleum ether, and the precipitate separated and dried. The product weighed 91 mg. and melted over a wide range 160–180°C. Anal: C, 64.95; H, 6.08; N, 13.57.

Desthiochetomin Distillation with Zinc Dust. Desthiochetomin (50 mg.) was intimately mixed with 2.0 g. of zinc dust. On heating, approximately 12 mg. of a yellow oil distilled out of the mixture. The oil had a strong skatole-like odor and gave intense color-reactions with glyoxylic acid and *p*-dimethylaminobenzaldehyde. The oil distilled in a Craig still at a bath temperature of 140°C. at 0.5 mm., but the distillate did not solidify. The substance was converted to the picrate, which was crystalline and melted at 148°C. This picrate had a composition similar to that of the picrate of a dimethyl or ethyl indole. Anal. Calc. for $C_{10}H_{11}N \cdot C_6H_3N_3O_7$: C, 51.33; H, 3.74; N, 14.98. Found: C, 51.99; H, 3.57; N, 15.67.

Desthiochetomin-Xanthydrol Derivative. A xanthydrol derivative, prepared like that of chetomin, was found to sinter at 115°C. and melt at 122°C. Analysis of the product indicated combination of one equivalent of chetomin with two of xanthydrol. Anal. Calc. for $C_{16}H_{19}O_4N_3 \cdot 2C_{13}H_8O$: C, 74.44; H, 5.76; N, 6.21. Found: C, 73.83; H, 5.10; N, 5.42.

Color Reactions. Because of the possibility of an indole nucleus in chetomin and desthiochetomin, certain color reactions given by this nucleus were investigated. Desthiochetomin gave a purple color in the Hopkins-Cole glyoxylic acid reaction and in the Ehrlich dimethylaminobenzaldehyde reaction. Chetomin also gave positive

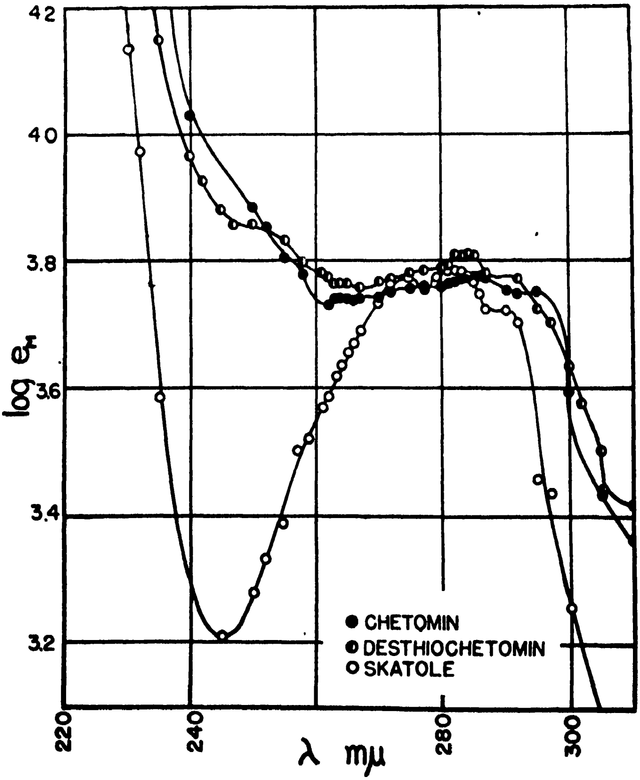

Fig. 1. Ultraviolet absorption spectra.

tests with these reagents, but the colors were slow in developing and less intense. The isatin test of Ciamacian and Zatti (13), which gives a purple color with indole, gave a carmine-red color with desthiochetomin and a green color with chetomin. The tests as a group confirm the probable presence of an indole grouping in desthiochetomin, but hint that the 1- or 2-position of the indole grouping may be blocked in chetomin.

Ultraviolet Absorption Spectrum. The ultraviolet absorption spectrum of solutions in absolute alcohol of skatole, chetomin and desthiochetomin is given in Fig. 1 (*cf.* 14). Skatole was found to have a triple peak in its absorption curve at 275, 283, and 290 mμ. Desthiochetomin showed a peak at 284 mμ, with shoulders at approximately 277 and 295 mμ. Chetomin showed a maximum at 287 mμ, and less marked shoulders near 278 and 297 mμ. The results tend to confirm the presence of an indole group in chetomin and desthiochetomin. A shift of the maximum toward longer wavelengths with increasing complexity of the compound was noted. Chetomin and desthiochetomin differ from skatole in showing strong absorption between 230 and 260 mμ; the presence of an additional chromophore is indicated.

SUMMARY

1. Chetomin has the probable formula $C_{16}H_{17}O_4N_3S_2$.

2. The functional groups of chetomin include 4 active hydrogen atoms, a methylimide grouping, probably a primary amide grouping, and possibly a thiosulfenic ester grouping.

3. Chetomin can be reduced with Raney nickel or zinc dust to yield a compound $C_{16}H_{19}O_3N_3$, designated desthiochetomin.

4. The presence of an indole nucleus in chetomin and desthiochetomin is indicated by degradation to an alkyl indole, by color reactions, and by the ultraviolet absorption spectrum.

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Ammonia Production by Kidney Slices of Normal, Acidotic and Alkalotic Rats

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INTRODUCTION

It has frequently been observed that, if some quantity of acid is given to an animal repeatedly over an experimental period, ammonia excretion by the animal increases for several days until it is approximately equal to the rate of acid administration (1). In considering this phenomenon, Lotspeich and Pitts (2) have advanced the suggestion that this "may represent a type of biochemical compensation expressed as an increase in the intracellular concentration of amino acid oxidase and glutaminase." This hypothesis seemed attractive since the arterial plasma concentration of amino acids (2) and glutamine (3) are unaltered in acidosis. The present report describes an attempt to compare the glutaminase and amino acid oxidase activity of the kidneys of normal rats with those of chronically acidotic and alkalotic rats.

EXPERIMENTAL

The experimental animals were adult male rats of the Vanderbilt strain (4), weighing 280–325 g. at the termination of the experiment. The control animals were fed a stock chow. Chronic alkalosis was induced by addition of 12 g. $NaHCO_3$/kg. to ground chow, while acidosis was obtained by addition of 3 g. NH_4Cl/kg. of chow. Feeding was continued for at least 10 weeks. At this time the animals were sacrificed by carotid section, the blood heparinized, and plasma taken for analysis. The means plasma HCO_3^- concentration in the normal rats was 22.6 m. eq./l.; in the acidotic rat 15.7 m. eq./l. and in the alkalotic rats 31.4 m. eq./l. No attempt was made to measure 24 hr. ammonia excretion by these animals. However, 6 hr. urine samples collected during the tenth week of feeding showed pH values of 4.7, 6.3, and 7.9 for acidotic, control, and alkalotic animals, respectively. Virtually no ammonia was present in the alkaline urine, while the ammonia excretion of the acidotic rats was about 7 times that of the control rats during this period.

For each experiment, one animal of each series was sacrificed, the kidneys removed immediately and kept moistened with 0.85% NaCl and slices then cut freehand with a straightedge razor. The slices were then weighed and incubated with shaking at 37°C. in 25 ml. Erlenmeyer flasks containing 4 ml. Ringer-bicarbonate medium equilibrated with 95% O_2–5% CO_2. The substrates were added in a concentration of 1.0 mg./ml. Ammonia was estimated colorimetrically by nesslerization following distillation *in vacuo* when amino acids were used as substrates. However, with glutamine as substrate, ammonia was estimated by direct nesslerization of the medium filtrate to avoid hydrolysis of the amide.

TABLE I

NH_3 Production by Kidney Slices of Acidotic and Alkalotic Rats

Incubation time	Substrate	NH_3−N formed		
		Control	Acidotic	Alkalotic
min.		γ	γ	γ
60	D-Alanine	146	154	153
60	L-Alanine	16	25	19
90	L-Methionine	19	11	
90	L-Leucine	19	18	
90	L-Glutamine	340	362	357
105	D-Alanine	264	259	257
105	L-Alanine	41	42	35
180	L-Alanine	40	49	24
180	L-Glutamic acid	112	130	112
180	D-Phenylalanine	157	220	172

Representative data are shown in Table I. In each case the ammonia produced by similar kidney slices from the same animal in the absence of substrate has already been subtracted and the values shown in the table represent ammonia production from the substrate.

ACKNOWLEDGEMENT

The author's thanks are due to the Duke University Research Council, the U. S. Public Health Service, and the Nutrition Foundation for their support of this work.

SUMMARY

From the data of Table I it is apparent that the previous history of these animals did not result in any consistent alteration of the rate at which their kidney slices produced ammonia from D or L amino acids or

glutamine. It would appear, therefore, that the progressive increase in ammonia excretion during experimental and clinical acidosis is not the result of an adaptive increase in the concentration of renal glutaminase or amino acid oxidases, but rather a functional response of the kidney to the progressively increasing severity of the acidosis.

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Carbon Dioxide Inhibition of Anaerobic Fumarate Formation in the Mold *Rhizopus Nigricans*[1]

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INTRODUCTION

Anaerobic formation of fumaric acid from glucose by a strain (No. 45) of *Rhizopus nigricans* has been reported in detail (1) together with evidence that the fumarate synthesis involves CO_2 fixation *via* the Wood-Werkman reaction, with the well-known subsequent transformations to fumarate.

$$\begin{tikzcd}
\text{Glucose} \arrow[d] & & & \\
\text{Pyruvate} \arrow[r, "+\,CO_2"] & \text{Oxalacetate} \arrow[r] & \text{Malate} \arrow[r] & \text{Fumarate}
\end{tikzcd}$$

It has been discovered that high CO_2 concentrations specifically inhibit fumarate formation under the above conditions and, furthermore, that oxalacetate decarboxylase activity of the mold is also inhibited by high CO_2 tensions, suggesting that fumarate formation is blocked at the pyruvate + CO_2 stage.

EXPERIMENTAL

Methods

Methodology was the same as in our previous report (1). Fairly homogeneous fungus mycelium suspensions were obtained by cultivating the organism on a shaking machine. The mycelium was harvested by filtration and washed with 1% HCl to dissolve excess $CaCO_3$ adhering to the mycelium, the carbonate having been present in the growth medium to neutralize the accumulated organic acidity. After a water wash, the mycelium was freed of excess water on a Buchner filter and the desired aliquots weighed directly on an analytical balance. The mycelium, so prepared, uniformly contained about 16% dry weight. The aliquots were transferred directly to the experimental vessels and dispersed to a fairly good homogeneous consistency in the particular liquids used. Aseptic precautions were unnecessary because the glucose solutions which were fermented rapidly became acid enough to suppress bacterial contaminations for the duration of the experiments.

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For a manometric study of the concomitant formation of CO_2 and alcohol with fumarate anaerobically see previous paper (1). The main chamber of the vessel contained 2.0 ml. water to which the mycelium was added, then dispersed with a probe. The atmosphere was replaced with natural gas (CH_4), and 1 ml. of 9% glucose added from the side arm after equilibration. The methane is a convenient inert gas diluent; it and nitrogen gas are equally useful. Tests insuring the absence of oxygen are described in the previous paper. This amount of carbohydrate is essential in order to obtain amounts of fumaric acid large enough to measure accurately with the analytical method employed (2). So much CO_2 formation was involved that mercury was used as the manometer fluid instead of Brodie's fluid. When the fermentation of sugar slowed down, as evidenced by the leveling off of CO_2 production, the vessel contents plus washings were transferred to conical centrifuge tubes and the glucose and fumarate analyses made on the supernatant.

Effect of CO_2 Absorption and Amount of Mycelium

The CO_2 inhibition was discovered when it was observed that anaerobic fumarate formation was weak when the experiments were conducted in small vessels as compared to larger vessels. The effect was traced to the size of the gas space; in the former the fermentation quickly led to a high CO_2 tension, while in large vessels the tension was naturally much lower. The amount of mold mycelium used is a second important factor in the anaerobic yield of fumarate.

A manometric experiment was set up to resolve these factors. Three different amounts of mycelium were compared, each in the presence and absence of NaOH as a CO_2 absorbent in the center well. The treatments without NaOH formed so much CO_2 that the excess had to be liberated several times during the run. Table I shows that both the amount of mycelium employed and the presence of CO_2 in the atmosphere influence the manner of glucose utilization insofar as fumarate formation is concerned. Both of the above factors appear to be critical. At all mycelium levels there was appreciably more fumarate formed (conversion) when the CO_2 derived from fermentation was absorbed from the atmosphere. Also, the smaller the amount of mycelium, the more efficient is the conversion of glucose to fumarate. This is especially striking in the treatments where CO_2 had been absorbed.

An unequivocal interpretation of this latter mycelium effect is not apparent, but a possibility is based on the idea of shunt metabolism (3).

That is, fumarate is formed at a maximum rate only as long as the carbohydrate concentration remains above a minimum level, which depends on the amount of mycelium. Separate experiments have shown that a definite optimum carbohydrate concentration for fumarate formation exists; 3% initial concentration proved to be optimum.

TABLE I

Anaerobic Fumarate Formation by Different Amounts of Mycelium in the Presence and Absence of a CO_2 Absorbing Agent

Amount of mycelium [a]	NaOH present	Glucose consumed	Fumaric acid	Weight conversion of glucose to fumaric acid
mg.		*mg./ml.*	*mg./ml.*	*per cent*
600	–	28.4	1.39	4.9
	+	28.4	2.50	8.8
200	–	24.0	2.03	8.45
	+	25.45	2.67	10.5
70	–	13.8	1.38	8.2
	+	9.7	1.78	18.4

[a] Moist weight

It is now possible to control the factors influencing reproducible results in securing high conversion yields; highest conversion yields are obtainable with small amounts of mycelium per unit of glucose available and in the presence of a CO_2 absorbent. The lower values for fumarate (Table I) in terms of mg./ml. in the case of 70 mg. mycelium is due, of course, to the fact that less glucose was consumed in the same time period than with the larger amounts of mycelium. The conversion percentages are the key figures in this experiment.

In view of the formation of anaerobic fumarate *via* CO_2 fixation (1) it might appear incongruous that CO_2 removal would favor fumarate formation instead of restricting it; but, as shown in the previous work, adequate CO_2 is always present intracellularly as a result of a concomitant alcoholic fermentation.

Effect of CO_2 Tension

A separate experiment was set up to study further the inhibition of fumarate formation by added excess CO_2 in the atmosphere. One hundred mg. amounts of mycelium were used in each case. The CO_2 tension was varied: the first was rendered CO_2-free by NaOH in the center well, one consisted of natural gas (CH_4, which contains about 1% CO_2), another consisted of 30% CO_2 in CH_4, and a final one of 100% CO_2. Strict anaerobiosis prevailed in all vessels. Suppression of fumarate formation was directly proportional to the CO_2 tension (Table II). Fumarate formation was entirely eliminated in a pure CO_2 atmosphere. One-third atmosphere of CO_2 reduced the fumarate yield to 25% of that in the CO_2-free control. The yield in 1% CO_2 was also inhibited to a small extent (9%), probably due to the fact that fermentation CO_2 raised the CO_2 tension as the fermentation developed. Glucose consumption was inhibited to a lesser degree by the higher CO_2 concentrations, but the main effect of CO_2 is a specific inhibition of the formation of the C_4 dicarboxylic acid. It is eliminated entirely, and a qualitative change in the metabolism of the fungus is thereby achieved. Under these same conditions, only the rate of the simultaneously occurring alcoholic fermentation is reduced.

TABLE II

Carbon Dioxide Tension and Anaerobic Fumarate Formation

Treatment	Glucose consumed	Fumaric acid	Weight conversion of glucose to fumaric acid
	mg./ml.	*mg./ml.*	*per cent*
No CO_2, NaOH in center well[a]	23.1 23.9	3.23 3.25	14.0 13.6
1% CO_2 in CH_4	20.0	2.52	12.6
30% CO_2 in CH_4[a]	15.8 18.1	0.60 0.58	3.8 3.2
100% CO_4[a]	10.1 11.0	0 0	0 0

[a] Values given for duplicate vessels.

CO_2 *Inhibition of Oxalacetate Decarboxylase*

Rhizopus nigricans mycelium (desiccated) has been shown to contain the enzyme responsible for CO_2 fixation *via* oxalacetate (OAA) (1), namely, oxalacetate decarboxylase. If the CO_2 inhibition of anaerobic fumarate formation results from an inhibition of the Wood-Werkman reaction (CO_2 + pyruvate $\longrightarrow$ OAA), CO_2 should inhibit the action of this enzyme. Fig. 1 shows that the action of this enzyme, displayed by decarboxylation of OAA, is substantially inhibited by a 100% CO_2 atmosphere. Sufficient phosphate buffer $\left(\frac{1}{7.5\ M}\right)$ maintained the pH between 6.5 and 7.0, which was also maintained in the zero CO_2 vessels. Thirty mg. dried material was used in each vessel, and 0.01 m*M* OAA furnished from the side arm. It will be noted that CO_2 exerted no significant inhibition of endogenous fermentation in the absence of OAA (Curves C and D), whereas there is a big differential in the treatments containing active enzyme plus OAA (Curves A and B). This differential cannot be ascribed to a suppression of the spontaneous decarboxylation of OAA because CO_2 had no significant effect on this reaction in the presence of boiled cells (Curves E and F). The true extent of the inhibition can be appreciated only after the appropriate controls are deducted from Curves A and B, the 60 min. values of which actually represent the total of CO_2 due to enzymatic decarboxylation, CO_2 due to spontaneous decarboxylation in the presence of boiled cell material, and CO_2 due to endogenous decarboxylation.

Thus, CO_2 formed enzymatically represented by Curve A $= 150 - (39 + 21) = 90$ µl. The CO_2 formed enzymatically represented by Curve B $= 75 - (36 + 24) = 15$ µl. Thus, the enzymatic CO_2 formed in the CO_2 atmosphere was only 15 µl as compared to 90 µl in the methane atmosphere, an inhibition of 83.3% due to the high concentration of CO_2.

Reversibility of CO_2 Inhibition

Data in Table III indicate that the inhibition is reversible. Here, aliquots of mycelium plus glucose were allowed to act (in duplicate on a shaker) for 1 day in a CO_2 and a CH_4 atmosphere, respectively. This is the first stage. The former produced no fumarate, the latter 7.15%. Glucose consumption was about equal in both treatments. The fumarate yield was not as high as is obtainable, owing to the fact that large amounts of mycelium were used. For the second stage, the mycelium in each flask was separated from the liquid on a Buchner filter, resuspended in 3% glucose solution and all treatments now exposed for one day to CH_4. Removal of the CO_2 in this second stage restored entirely

TABLE III

Reversal of CO_2 Inhibition

	Mycelium treatment			
	First stage CO_2	Second stage CH_4	First stage CH_4	Second stage CH_4
	mg./ml.	*mg./ml.*	*mg./ml.*	*mg./ml.*
Glucose consumed	23.0	25.4	24.4	22.0
Fumaric acid	0	2.38	1.75	1.83
Per cent conversion	0	9.4	7.15	8.2
Final pH	3.8	2.6	2.6	2.7

the fumarate-producing capacity of the mycelium. The final pH values clearly reflect the acid formation.

Absence of Other Acids

Analysis of the culture filtrates, devoid of fumarate through CO_2 inhibition, shows less than 5% of the total acidity formed normally, this being almost entirely lactic acid, which also is formed normally. Traces of malic acid are indicated, but no succinate. The main product of the sugar is ethyl alcohol. These findings indicate that failure to produce fumarate is not due to inhibition of conversion reactions among the C_4 dicarboxylic acid precursors of fumarate. They likewise suggest that the inhibition deals with a reaction preceding C_4 dicarboxylic acids, in particular, that governing C_4 synthesis, namely $C_3 + C_1$ condensation.

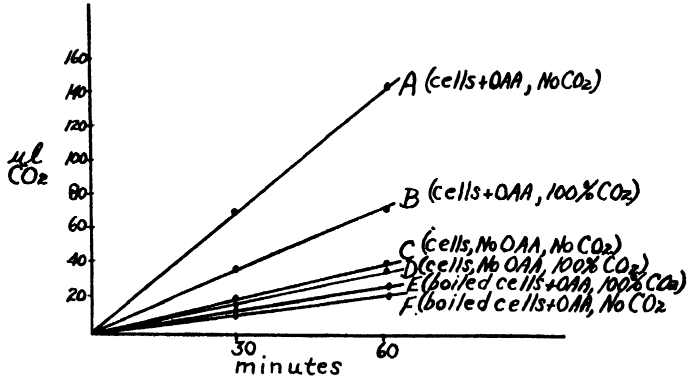

FIG. 1. CO_2 inhibition of oxalacetate decarboxylase

DISCUSSION

An exact understanding of the nature of the CO_2 inhibition cannot be formulated at present. Any hypothesis will have to reconcile the fact that CO_2 is one of the reactants in the synthesis of C_4 dicarboxylic acids *via* the Wood-Werkman reaction and, hence, *via* mass action would, in higher concentrations, be expected to increase the rate of synthesis, whereas, in reality, inhibition is observed. Obviously, then, CO_2 has a second effect (inhibitory) on oxalacetate decarboxylase, and moreover, it is relatively specific for this enzyme in view of the fact that the numerous enzymes of the zymase complex remain active under CO_2 tensions which entirely suppress fumarate formation. At least, in this particular system, this enzyme is much more sensitive to CO_2 than the numerous enzymes of the zymase complex.

The fact that the CO_2 inhibition appears selectively against an enzyme which normally metabolizes CO_2, and that the inhibition is reversible, raises the possibility of some sort of a block, possibly of the competitive type, between CO_2 and the carboxyl group at the site of formation of carboxyl groups on the enzyme surface. Bicarbonate inhibition of glutamic acid conversion to glutamine in *Lactobacillus arabinosus* (4) is, perhaps, another example lending itself to this kind of interpretation. However, this is only speculation.

The CO_2 inhibition with living mycelium was obtained at pH 3–4, that is, where bicarbonate cannot exist. No sign of inhibition was observed in 0.003 *M* $NaHCO_3$ at neutral pH with a 5% CO_2 atmosphere, the usual conditions employed in manometry. Possibly higher CO_2 concentrations would be effective also at neutral pH vlaues. However, it may be that CO_2 penetrates the cell membrane *per se* and not as bicarbonate ion, or that the effect is obtainable only in the lower pH range. Karow (5) obtained CO_2 inhibition of *Aspergillus wentii* growth by 15% CO_2 at pH 2.0 but not at pH 6.5, results similar to the above. Differential biological activities of CO_2 and bicarbonate due to membrane permeability are known (6,7). Also, recalculation of Table 8 of Karow's thesis on *Aspergillus wentii* (5) reveals a reduction in citric acid synthesis by elevated CO_2 tensions, a fact in accordance with the inhibition of the Wood-Werkman reaction, which is followed in the synthesis of citrate by fungi (1,8).

The Wood-Werkman reaction appears to be of greatest importance in the economy of heterotrophic cells, and the above-demonstrated inhibition may partially explain the retardation of growth of biological systems by elevated CO_2 tensions. Fungi in particular appear subject to CO_2 inhibition (9,10,11). One wonders whether the intracellular concentration of CO_2 in biological systems may function as a regulatory mechanism in certain phases of metabolism.

SUMMARY

Anaerobic formation of fumaric acid from glucose by Strain No. 45 of *Rhizopus nigricans* is specifically suppressed by high tensions of CO_2. Also influencing fumarate formation, but to a lesser extent, is the amount of mycelium employed per unit of glucose available; yields are higher the smaller the amount of mycelium, this effect being ascribed to shunt metabolism. The CO_2 inhibition is reversible, and fumarate formation is restored when the CO_2 is removed. Oxalacetate decarboxylase in cell preparations of *R. nigricans* is inhibited by high CO_2 tensions. These points provide additional implication of the Wood-Werkman reaction in the synthesis of fumarate anaerobically by this mold. The significance of the CO_2 inhibition of this reaction in biological systems in general is discussed.

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The Addition of Cyanide to Sugars [1]

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INTRODUCTION

A former paper (1) described the use of Kiliani's cyanhydrin reaction as a quantitative measure of reducing groups in sugar molecules. The cyanide remaining after a 24 hr. period was determined by titration with silver nitrate in ammoniacal solution.

The scope of this reaction required study, since the addition of cyanide to ordinary aldehydes does not usually go to completion. Several aromatic aldehydes we tried showed no addition even after 72 hr.

The work reported in this paper shows the rates of reaction for various sugars. The rates vary considerably. Some of the sugars, like arabinose, react quickly, while others, like glucose, react more slowly. An increase in temperature, as expected, effects an increase in the rate of reaction for all sugars.

Change of pH influences the rate of reaction markedly. Almost all of the sugars react at the usual pH for the quantitative method (9.1). A drop in pH slows the rate. At acid pHs the reaction, for all practical purposes, comes to a halt.

The background of the Kiliani reaction includes a long history. From its beginning (2), liberal use has been made of it in synthetic work for the buildup of sugars. Hudson recently (3) reviewed fully the general usefulness of this interesting reaction.

EXPERIMENTAL

The conditions employed were essentially the same as those set forth earlier (1). Better concentrations might have been chosen for rate studies, but the main purpose of this work was to choose the conditions best suited for quantitative results.

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[1] Aided by a grant from the University Research Council, University of Nebraska.

All solutions were brought to the required temperature before the reaction was started. Two-tenths g. of the anhydrous sugar (1.11 millimoles) were dissolved in 10.00 ml. of 0.09 *N* acetic acid in a 125 ml. glass-stoppered Erlenmeyer flask. Ten ml. of 0.2 *N* KCN were pipetted into the solution and mixed. The stoppered flask, sealed with glycerol, was placed in a constant temperature bath at 25°C. (or 40°C.) for the required time. The excess cyanide was then titrated with 0.1 *N* $AgNO_3$ in the usual manner, after adding approximately 10 ml. of 6 *N* NH_4OH and 0.2 g. of KI. The cyanide consumed was obtained by subtracting the titration from the blank and multiplying by 13.

The conditions in the foregoing paragraph give a pH of 9.1. During the course of the experiment there was little change in pH, the final value after completion was still 9.0.

Lower pH values were obtained by adding 0.2 *N* acetic acid in place of 0.1 *N* and by adding water to bring the total volume to 20 ml. As an example, for a pH of 7.4 the quantities used were 8.75 ml. 0.2 *N* acetic acid, 1.25 ml. water, and 10.00 ml. of 0.2 *N* KCN.

RESULTS

Fig. 1 gives the rates of reaction for 4 monosaccharides: glucose, arabinose, galactose, and 2-keto-D-gluconate. These 4 were chosen to show the wide differences that occur. Arabinose and galactose reacted completely in a few hours. The same is true for fructose, mannose, and xylose, whose curves parallel so closely those of galactose and arabinose

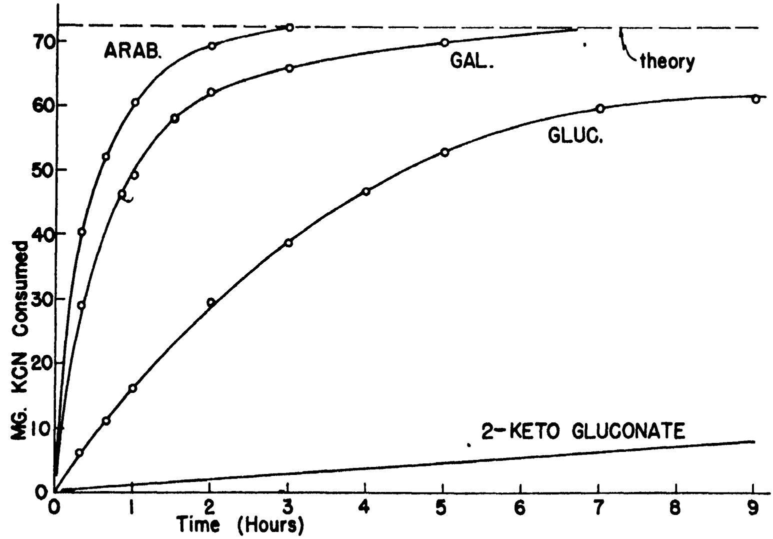

FIG. 1. Rates of cyanide consumption (calculated as KCN) for 4 representative monosaccharides, arabinose, galactose, glucose, and 2-keto-D-gluconate; temperature 25°C.; pH 9.1.

that it seemed unnecessary to show them. Glucose, on the other hand, reacted much more slowly. In 5 hr. it had consumed about 70% of the theoretical amount of cyanide. In 18 hr. the reaction was complete.

2-Keto-D-gluconate was the slowest of all the sugars thus far encountered. It showed very little consumption of cyanide in 10 hr. Finally, after 72 hr., it did take up the theoretical amount. This sluggishness of reaction does not extend to other 2-keto-sugar acids, since a sample of 2-keto-D-galactonate gave a rate similar to that of galactose and reached theory in a few hours.

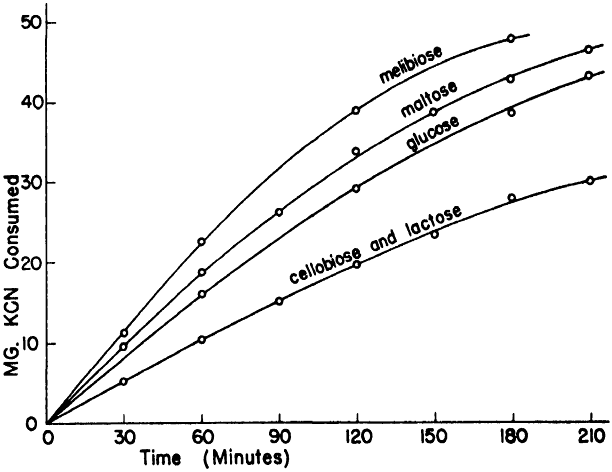

FIG. 2. Rates of cyanide consumption (calculated as KCN) for the reducing disaccharides, maltose, lactose, cellobiose, and melibiose, as compared to glucose; temperature 25°C.; pH 9.1.

An interesting point developed from the 4 common reducing disaccharides. They gave rates of reaction similar to glucose (Fig. 2). The rates on the same molar concentration (1.11 millimoles) are slow compared to the bulk of the monosaccharides. But those having a β-saccharide linkage (lactose, cellobiose) gave noticeably slower rates than those with an α-linkage (maltose, melibiose). We have not yet been able to test the extent of this relation on other reducing disaccharides because of the difficulty of obtaining these rare sugars. There is no obvious explanation for the difference between the disaccharides, since it would be supposed that the configuration of the saccharide coupling would have little effect on the reactivity of the reducing carbon.

Any explanation for the differences in rates of reaction from sugar to sugar would be purely speculative. A clue to the reason, however, is obtained from an analysis of the results for the order of reaction. Plotted by the conventional methods for rate constants, a straight line results for galactose when time is plotted against log $a\text{-}x/b\text{-}x$ (Fig.3). This would indicate a second order reaction.

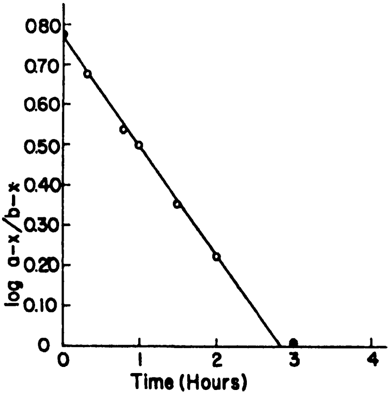

FIG. 3. Log $a\text{-}x/b\text{-}x$ for galactose concentration plotted against time in the cyanide reaction; temperature 25°C.; pH 9.1.

A plot of glucose, however, gives a reaction order of the first order rather than of a second. The same is true for lactose. At first, this was thought to be due to the slowness of these sugars and to the reaction assuming the characteristics of a pseudo first order, since the total cyanide exceeds the sugar concentration. But at 40°C. the rate of reaction for glucose equals that for galactose at 25°C. (see Fig. 4) and the plot of the results at 40°C. still shows a first order reaction for glucose.

To add to the complexity, a concentration of 1.95 millimoles for both cyanide and glucose shows the character of neither a first nor a second order reaction.

Most likely, the reaction between cyanide and sugar runs the course of consecutive reactions. The sugar surely must have to change to the free aldehyde form, and it is fairly likely that the cyanide must be in the form of hydrocyanic acid. There would, then, be two equilibria: sugar $\rightleftharpoons$ aldehyde form, and $KCN + H_2O \rightleftharpoons HCN + KOH$. At pH 9 the HCN/KCN ratio is approximately 1 (as calculated from the Henderson-Hasselbach equation). Since the hydrolysis of a salt is very rapid, the HCN concentration should not limit the reaction at any time. The limiting factor is then the concentration of the aldehyde form of the sugar. The differences in rates of reaction can then be accounted for by the varying ease of shift for sugar to aldehyde.

The effect of temperature is the conventional effect. At 40°C. the rate for glucose approximately doubles over that at 25°C. (see Fig. 4).

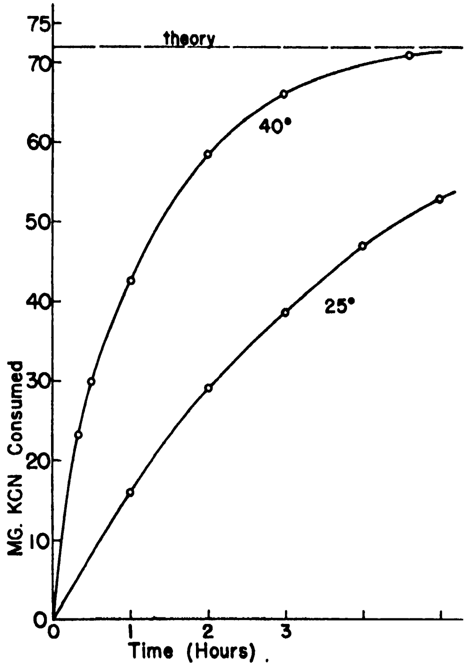

FIG. 4. Rate of cyanide consumption (calculated as KCN) for glucose at 40°C. compared to the rate at 25°C.; pH 9.1.

For quantitative work on the Kiliani reaction, the most favorable temperature is 40°C. At this temperature the loss of cyanide (by hydrolysis to formate) is small, about 3 parts per 1000 in 4 hr. The sugars react with reasonable speed: glucose goes to completion in 5 hr., whereas at 25°C. it requires 18 hr. At higher temperatures the loss of cyanide becomes too large for acceptable results, although blanks can be used to correct for the error.

A drop in pH slows up the Kiliani reaction and further emphasizes the fact that the sugar and not the cyanide holds the key to the reaction

(Fig. 5). At pH 7.4 the HCN/KCN ratio is about 40, yet the rate for arabinose slows down to ⅕ of its value at pH 9.1. At 6.5 the rate becomes too slow to have much significance. This shift in rate correlates with the abilities of sugars to change from the hemiacetal form to the free aldehyde form: a reaction favored by alkali and hindered by acid.

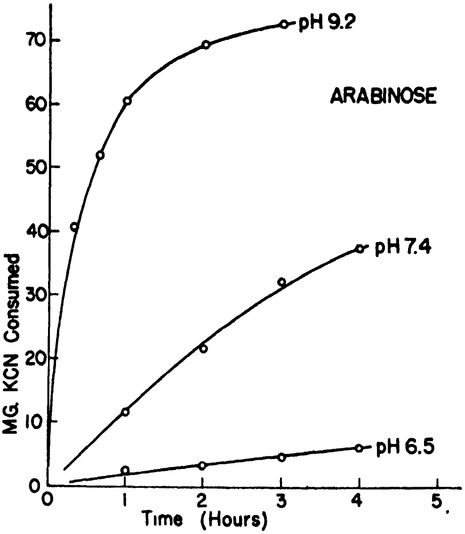

FIG. 5. Effect of pH change on the rate of cyanide consumption for arabinose; temperature 25°C.

SUMMARY

Sugars react with cyanide at varying rates. Some, like arabinose, react completely in a few hours; others, like glucose, require much longer. Disaccharides show characteristic rates depending on the nature of the saccharide coupling.

The reaction appears to be complex and does not follow a simple order of reaction for all sugars. Glucose reacts as a first order reaction, whereas galactose reacts as a second order.

A rise in temperature speeds up the reaction, and a decrease in pH slows it. At acid pHs the rate for the common sugars is almost zero.

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The Minimum Size for the Structural Unit of Pectin

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INTRODUCTION

Ehrlich (1,2,3,4), in his studies on pectin, isolated a product he believed to be the fundamental galacturonic acid polymer of pectin. This product, *Pektolsäure*, was considered to be a 4-membered galacturonic acid ring structure. *Pektolactonsäure* was obtained from the latter by the opening of one of the saccharide linkages. Morell, Baur and Link (5) refuted Ehrlich's ring concept and established the linear polymeric structure of the fundamental unit of pectin. They obtained, by the action of HCl-CH_3OH on pectic acid, an apparently homogeneous methylglycoside of polygalacturonic methyl ester (MPME) which consisted of 8–10 galacturonic acid units in glycosidic linkage. They concluded that the structural unit of citrus pectin must contain a linear polymerized galacturonic acid anhydride with a *minimum* size of 8–10 units. Baur and Link (6) subsequently obtained a 70% yield of the same or a similar methylglycoside of polygalacturonic methyl ester by similar treatment of Ehrlich's *Pektolsäure*. The *Pektolactonsäure* yielded only 12% of the same residue. They concluded that Ehrlich's preparations were not tetragalacturonic acid anhydrides, since they gave rise to polymers containing approximately 10 condensed galacturonic acid units. Furthermore, from the fact that on alcoholysis the *Pektolsäure* yielded 70% of the resistant polygalacturonide, whereas the *Pektolactonsäure* yielded only 12% of the same residue, it was evident that these preparations differ radically in molecular size.

This report deals with an extension of the work of Morell, Baur and Link (5). At a considerably later time after their publication it was shown in this laboratory (7) that pectic substances have a pronounced tendency to retain ethanol of precipitation even under drying conditions which are usually considered adequate for analytical purposes, thus giving erroneous Zeisel methoxyl values. The retained alcohol can be removed by humidification. Since Morell, Baur and Link (5) used ethanol precipitation for the purification of the MPME and some of its derivatives, *i.e.*, the free acid obtained from saponification, the methylglycoside of polygalacturonic acid (MP), and since they deduced its size from Zeisel methoxyl determinations, both terminal glycosidic and ester methoxyl, it was decided to reinvestigate the analysis of this substance and ascertain whether retained ethanol had affected the analysis. The results reported here make it appear that those of Morell *et al.* were influenced by retained ethanol. The average molecular weight of preparations made in this laboratory was approximately 5000, based on both chemical analysis and physical measurements. Diffusion measurements showed a high degree of heterogeneity. The heterogeneity was found to be due to about 15% of very small molecules; the remaining apparently homogeneous 85% had a molecular weight of approximately 6000, corresponding to 32 galacturonic anhydride residues. Thus, the postulated structural unit of pectin has been successively raised from 4 to at least 8–10, and now to at least 32 galacturonic acid units.

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METHODS AND MATERIALS

Clark's (8) modification of the Viebock and Schwappach method was used for the determination of methoxyl. The determinations of uronic anhydride were carried out according to Dickson, Otterson and Link's (9) modification of the Lefevre and Tollen's method. All uronic anhydride values are corrected for methoxyl, ash, and moisture. Samples were humidified by the procedure recommended previously (7). Osmotic pressure determinations were made with the apparatus described by Bull (10). The diffusion analyses were made at 28.8°C. with 1% solutions in phosphate buffer of pH 6.5 and ionic strength 0.1. A Tiselius electrophoresis apparatus with a Svensson-Philpot optical system (11) was used for these experiments (without applied voltage). In order to analyze the Svensson-Philpot photographs, these were enlarged photographically with the emulsion in contact with a negative copy (transparent crossed rulings on an opaque background) of coordinate paper. This device makes it possible to treat the photographic records by the methods of Lamm (12) and Gralén (13). The enlargements were also measured with dividers and a planimeter to find diffusion coefficients ($D_{o,A}$) without depending upon the coordinate grid (14).

The methylglycoside of polygalacturonic methyl ester was prepared from citrus pectic acid and apple pectin (5). The pectic acid and pectin were first purified with hot 70% ethanol according to the method of Morell and Link (15) and had, after purification, a uronic anhydride content of 90.1 and 90.3%, respectively. After the 90 hr. reflux with HCl-CH_3OH the insoluble material was centrifuged off, washed with anhydrous CH_3OH and acetone, and then dried in a vacuum oven at 70°C. for 18 hr. Yields of 58 and 59%, respectively, were obtained. The MPME was purified by solution in water, filtration through Celite, precipitation with 4 volumes of ethanol, and washing with ethanol. The precipitates were then dried in a vacuum oven at 70°C. for 18 hr.

A water solution of a part of the citrus MPME was dialyzed in Cellophane tubing against distilled water in the cold for 11 days with frequent changes of water, after which 40% of the material remained in the tubing. The solution was then lyophilized.

MPME from both sources was saponified by the addition of 30 ml. of 5 *N* NaOH to 10 g. of the material dissolved in 200 ml. of water. After standing at room temperature for 1.5 hr., the pH was adjusted to 1.0 with dilute HCl, and, after standing for 30 min., an equal volume of ethanol was added and the precipitate centrifuged off. The precipitate was taken up in water (approximately 1200 ml. was necessary for solution) filtered through Celite, and upon the addition of an equal volume of ethanol only a slight opacity resulted. It was found necessary to add a small amount of HCl solution to obtain precipitation. The precipitate was centrifuged off, washed with ethanol and dried in a vacuum oven at 70°C. for 18 hr. A yield of 93% was obtained.

RESULTS

The analytical results are reported in Table I. It can be seen from the values for the per cent methoxyl that the MPME and MP prepared

TABLE I

Analysis of the Methylglycoside of Polygalacturonic Methyl Ester and the Methylglycoside of Polygalacturonic Acid

Material	Per cent uronic anhydride[a]	Per cent OCH_3 (Zeisel)			Titratable acidity calc. as per cent OCH_3[c]
		Literature (5)	Before humidification	After humidification[b]	
MPME (Apple)	96.0	—	18.0[a]	16.6	0.2
MPME (Citrus)	97.7	18.0±1.0[a]	17.4[a]	16.2	0.5
Dialyzed MPME (Citrus)	97.7	—	—	15.7	0.5
MP (Apple)	97.1	—	4.6[a]	0.85	—
MP (Citrus)	98.1	2.2[a]	4.3[a]	0.85	—

[a] Dried to constant weight over P_2O_5 at 100°C. in an Abderhalden dryer at low pressure.

[b] Results calculated to a moisture, ash-free basis, the samples having been dried after humidification.

[c] Results obtained on samples after humidification and redrying.

from citrus pectic acid and apple pectin all retain alcohol which, as in the case of pectin (7), cannot be removed by drying at 100°C. at low pressure. Further, the citrus MPME result agreed with the reported values (5), whereas the citrus MP was higher. After humidification and redrying, the values for the per cent methoxyl were lower, and even if the free carboxyl groups (calculated as per cent methoxyl) are included, the values are considerably lower than those reported by Morell *et al.* (5). The terminal glycosidic methoxyl value of 0.85% for the MP would correspond to a molecular weight of 3700 for the free acid, and 4000 for the MPME, or a unit consisting of approximately 21 galacturonic anhydride residues. Such a unit when completely esterified would contain 17.0% methoxyl. The methoxyl content of MPME reported in Table I is in good agreement with this value, thus suggesting that the minimum size of the structural unit of pectin, on the basis of analysis which avoids retained alcohol, is more than twice that suggested by Morell, Baur and Link (5).

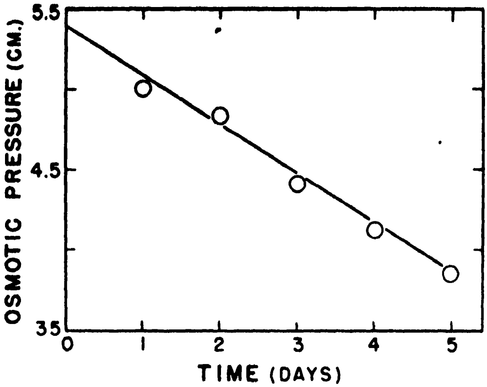

FIG. 1. Osmotic pressure of the methylglycoside of polygalacturonic methyl ester as a function of time.

Physical measurements showed citrus MPME to be heterogeneous, although the number average molecular weight calculated from these measurements is in good agreement with those calculated from chemical analysis. Osmotic pressure measurements were confused by dialysis of at least part of the material. The osmotic pressure decreased with time, as shown in Fig. 1, but, since this decrease was almost linear, extrapolation to zero time would appear to be justified for the determination of the correct osmotic pressure. The number average molecular weight on the basis of the zero time pressure is 4700.

Diffusion measurements of citrus MPME were made and analyzed by the methods cited. $D_{o,h}$, the diffusion coefficient determined from the maximum height and area of each curve, was 1.16×10^{-6} cm.^{2}/sec., referred to distilled water at 20°C. ($D_{o,h}$ is the square of the reciprocal of the weight average of the square roots of the reciprocals of the diffusion coefficients of all diffusing species.) Owens[3] of this Laboratory has determined the frictional ratio, f/f_o, for pectic substances from viscosity data in accordance with the treatment outlined by Oncley (16) and found f/f_o for MPME to be 1.60. The average molecular weight was computed from these values of $D_{o,h}$, f/f_o, and the density 1.5,[4] using the equation

$$M = \frac{R^3T^3d}{162\pi^2N^2D^3\eta^3\,[f/f_o]^3}.$$

The value obtained, 5800, although an average value of a different sort, is comparable with that given by osmotic pressure.

In measuring free diffusion, we (unlike Longsworth (14)) regularly encounter appreciable disturbances in forming the boundaries. Therefore, in determining $D_{o,h}$, the quantity A^2/H^2 (times a proportionality constant determined by the enlargement factor) is plotted as a function of time. In this expression A is the area under a curve and H is its maximum height, both measured to the middle of the line. The proportionality constant is $l^2/4\pi$, where l is the degree of linear enlargement from the cell to the measured print. When this graph is made it is seen that A^2/H^2 is nearly linear with time, but that the lines do not pass through the origin. Therefore, the rate of change of $(A^2/H^2)(l^2/4\pi)$ with respect to time is taken as $D_{o,h}$. This is equivalent to taking the intersection of the graph with the time axis as determining the zero time. The diffusion coefficient found in this way is the value approached by the diffusion coefficients computed from the measured times as these times become large. In practice, the slope and intercept of the line best fitting the experimental points is found by the method of least squares.

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[3] Private communication from Dr. H. S. Owens.

[4] Palmer *et al.* (17) of this laboratory found that the densities of all pectic substances were within 1% of 1.50.

Similarly, in determining the weight average diffusion coefficient, $D_{2,0}$, by the method of moments, the quantity $\sigma^2/2$ plotted against time indicates a zero time different from the instant the boundary was formed. σ^2 is the second moment of the diffusion curve referred to unit area. Nevertheless, the slope of the curve is used to determine $D_{2,0}$, and was 1.08×10^{-6} cm.^{2}/sec.

A reason for determining both of these average diffusion coefficients is that the ratio $D_{2,0}/D_{o,h}$ is an index of the homogeneity of the diffusing substance. The diffusion coefficients are the same for an ideal homogeneous material, but $D_{2,0}$ is always larger for a mixture of ideally diffusion substances. In other words, the curve given by an ideal homogeneous substance has the same form as the normal probability curve, while the curves of ideal mixtures are sharper. Indeed, it is possible to determine the ratio of $D_{2,0}/D_{o,h}$ for a single experimental curve without reference to time and without actually determining the coefficients or making use of enlargement factors. In our first experiments, this ratio was observed to change from 1.5 to 0.9 in an interval of about thirty hr. A related circumstance is that the zero times found by the two methods did not coincide. In studying these effects, it was noticed that the area under the curves, when referred to the same slit angle, decreased by 15% during the experiment. Since the area is proportional to the change in concentration across the boundary, this observation suggested that the change in form and the behavior in dialysis might be due; in part, to rapid diffusion of a low molecular weight component, associated with an essentially uniform principal component of diffusion coefficient near 1.1×10^{-6} cm.^{2}sec.$^{-1}$ referred to water at 20°C. and making up about 85% of the whole.

Therefore a sample of citrus MPME was dialyzed against distilled water, lyophilized, and reanalyzed. The remaining 40% gave an osmotic pressure, constant during the period of observation, corresponding to a molecular weight of 5700 ± 100[5] or a unit of 30 galacturonic acid residues. The theoretical methoxyl content for such a unit, including both ester and terminal glycosidic methoxyl, would be 16.2% when completely methylated. The value obtained (Table I) was 16.2% when methoxyl value equivalent to the free acid groups was added to the actual Zeisel result.

A diffusion analysis of the dialyzed MPME was made. Although the zero time was greater than in the first experiments, the ratio $D_{2,0}/D_{o,h}$ was 1.1 after one hr., as compared with 1.5 for the same time in the study of the undialyzed MPME. After 40 hr., the ratio was 1.0. The areas of the curves show no trend with time. $D_{o,h}$ was found to be 1.10×10^{-6} cm.^{2} sec.$^{-1}$ referred to water at 20°C. The corresponding $D_{2,0}$ was

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[5] This value was obtained from 4 determinations at 2 concentrations: 0.1 and 0.25%.

1.13×10^{-6}, computed from the value of σ^2 at 146,800 sec., applying the zero time correction determined from the data for $D_{o,h}$, or 1.10×10^{-6}, computed from the difference in σ^2 at 4800 sec. and 146,800 sec. The diffusion coefficient 1.10×10^{-6} corresponds to molecular weights of 6810.

The chemical, osmotic, and diffusion data, together, make it appear that the MPME is predominantly a single substance of molecular weight between 5000 and 7000, corresponding to the methyl ester of a polygalacturonide consisting of about 32 galacturonic anhydride residues. This unit is believed to represent a real structural unit of pectin (other than galacturonic acid) and larger than others hitherto proposed.

Discussion

The fact that the relatively drastic treatment employed in the preparation of the methylglycoside of polygalacturonic methyl ester resulted in essentially a homogeneous material, in yields of as much as 50%, may be considered as evidence that this unit must be a distinct part of the pectin molecule. The unit may have its origin by virtue of hydrolysis (or alcoholysis) of bonds other than the usual 1,4-glycosidic bonds joining the galacturonic acid residues. The bond being hydrolyzed to give rise to the unit must be weaker than the predominant glycosidic bond; otherwise, the reaction product would be very inhomogeneous. The uronic anhydride contents of the initial and the final products may be considered as evidence in favor of this postulate. The original purified pectic acid had a uronic anhydride content of 90%, whereas the reaction product (MPME) was composed of essentially all uronic anhydride (98%). This postulate would also necessitate that the non-uronide material constitute an integral part of the pectin molecule. McCready *et al.* (18) have found that the last 10% of the non-uronide material in pectic or pectinic acids cannot be removed except by methods involving hydrolysis of primary bonds, thus indicating that this non-uronide material is an integral part of the pectin molecule.

Summary

Previous chemical analysis of the methylglycoside of polygalacturonic methyl ester, which was found to be the structural unit of pectin by Morell, Baur and Link (5), were shown to have been influenced by retained alcohol. After the retained alcohol had been removed, methoxyl determinations, both terminal glycosidic and methyl ester, showed the material to consist, on the average, of molecules containing 21 galacturonic anhydride residues corresponding to a molecular weight of 4000.

Physical measurements showed the methylglycoside of polygalacturonic methyl ester prepared from citrus pectic acid to be heterogeneous, of course, although the average molecular weights calculated from these measurements were in good agreement with that calculated from chemical analysis. The average molecular weight calculated from osmotic pressure determinations extrapolated to zero time (since some material dialyzed with time) was 4700, while that calculated from the diffusion coefficient $D_{o,h}$ was 5800. However, the area under the diffusion curve decreased during the course of the experiment to such an extent that, taken together with the progressive change in the shape of the curve, the data suggested that the material was contaminated with a small amount of a fast-diffusing component.

After dialysis of the methylglycoside of polygalacturonic methyl ester, constant osmotic pressure measurements were obtained, corresponding to a molecular weight of 5700. Chemical analyses were in agreement with this value. The ratio, $D_{2,0}/D_{o,h}$, changed relatively little during the course of the diffusion experiment on the dialyzed material, and no progressive change in area occurred. An average diffusion coefficient of 1.10×10^{-6} cm.^{2}sec.$^{-1}$ referred to water at 20°C. was determined. The average molecular weight calculated from diffusion data was 6800.

Therefore, the methylglycoside of polygalacturonic methyl ester is essentially a homogeneous material of a molecular weight of approximately 6000, corresponding to the methyl ester of a polygalacturonide consisting of about 32 galacturonic acid anhydride residues, representing the largest reported structural unit of pectin. The unit possibly has its origin in its hydrolysis from the integral non-uronide material of pectin.

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A New Method for the Preparation of Uricase and the Effect of Uricase on the Blood Uric Acid Levels of the Chicken [1]

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INTRODUCTION

It has long been recognized that gout is a metabolic disorder in which the blood uric acid is usually elevated. The mechanism by which this elevation of blood uric acid occurs has not yet been established. It is quite possible that the finding is only a sign of a more fundamental disorder and that, were a method found of maintaining the blood uric acid level withing normal limits in a gouty patient, the degenerative process would still continue. However, as the elevation in blood uric acid values is the one laboratory sign which, together with the clinical findings, establishes a diagnosis of gout, it would seem logical to attack the problem of alleviating the symptoms of gout and the formation of tophi by attempting to lower the blood uric acid to normal levels by the injection of uricase. However, three dfficulties arise: (1) the destruction of the enzyme by the blood proteinases; (2) the possible formation of protein sensitization through repeated injections of enzyme; and (3) the probable formation of an antienzyme.

Uricase, an enzyme present in the tissues of certain animals but not in humans, oxidizes uric acid to allantoin. It seems possible that the injection of this enzyme into humans might result in a lowering of an elevated blood uric acid. This possibility was investigated by Oppenheimer and Kunkel (1,2) by injecting uricase intramuscularly into chickens fed a meat diet to produce an abnormally high blood uric acid level. They reported a fall in the plasma uric acid level ranging from 17 to 79% 1 hr. after injection of uricase. The uricase used by these workers was not in solution but in a colloidal suspension.

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[1] This work was supported by a grant given to Dr. Seymour Gray by the Nutrition Research Foundation. Dr. Gray left for military duty when these experiments were undertaken.

The first step in the solution of this problem was the preparation of an enzyme soluble in water and of sufficient purity as to lower the possibility of producing dangerous protein sensitizations on repeated injections. The second step was to repeat Oppenheimer's observations, since no further confirmation of these has appeared.

We present in this paper a method for obtaining uricase preparations of high activity and the results obtained when these preparations were injected into chickens.

EXPERIMENTAL

Studies on the Purification of Uricase

The two most recent methods of uricase preparation (3,4) start with an acetone precipitation of liver and end with water-insoluble colloidal suspensions. The preliminary acetone precipitation was found to destroy a large amount of enzyme, probably through protein denaturation. It was decided, therefore, to start with different procedures less harmful to the proteins. The procedure is described below; the organ chosen was the kidney (beef).

I. Defatted beef kidney (1860 g.) was ground in a meat grinder and then transferred to a mechanical mortar in which the mixture containing the kidney pulp, 45.2 g. Na_2CO_3, 37.6 g. $NaHCO_3$, 170 cc. toluene, and 1560 g. sand were ground continuously for 7.5 hr. at room temperature. This material was then incubated at 38°C. for 48 hr. and extracted with 2800 cc. 0.2 *M* borate buffer, pH 8.93, by grinding in a mechanical mortar for 1 hr. After straining this material and re-extracting the residue with 950 cc. of borate buffer as above, 2600 cc. of soluble uricase were obtained. This material was now dialyzed against running cold distilled water for 10 hr. = 3400 cc. of solution. Purification: 110 times; yield: 4.65 g. protein N; activity: W[2] = 104.5.

II. To 3.4 l. of dialyzate were added 3.2 l. of $Zn(OH)_2$ gel (prepared as described below). The gel was stirred in slowly for 1 hr. with constant mechanical stirring and the precipitate thus formed allowed to stand for 3 hr. at 4°C. The precipitate was then removed by centrifugation and eluted by grinding in the mortar with 1500 cc. of 0.1 *M* phosphate buffer, pH 7.5. The elution should be carried out in small portions over a period of 2 hours in the cold room. Purification: 340 times; yield: 1.02 g. protein N; $W = 148$.

III. One and one-half l. of eluate were then treated with 150 cc. γ-$Al(OH)_3$ gel (Willstätter). The adsorption was carried out over a period of 2 hr. at 3°C. The resulting precipitate was centrifuged off and discarded. Purification: 1000 times; yield: 0.46 g. protein N; $W = 940$.

At this point the solution is transparent and of very light brown appearance. It may be kept in this state for about 2 weeks without loss of activity, or it may be lyophilized, in which case it can be kept for several months or longer. The solution shows no precipitate when exposed to a centrifugal field of 12,000 *g*, and gives a Tyndall effect. This preparation, when used in uric acid analysis, gives no appreciable blank values with the phosphomolybdic reagent, as contrasted with other preparations and thus is particularly well suited for analytical purposes.

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[2] $W = mm^3.\ O_2$ uptake/mg. N/hr.

Further purification may be achieved by precipitating the enzyme as a "nucleoprotein," using nucleic acid and then removing the nucleic acid with salmine. The nucleic acid used was a purified commercial yeast nucleic acid (sample obtained from A. D. Mackay, New York).

IV. To 150 cc. of the enzyme solution obtained from step III were added 4 g. of commercial nucleic acid (solid). The supernatant obtained after centrifugation was discarded. The precipitate, obtained after standing for 1 hr. in the cold room, was dissolved in 70 cc. 0.1 *M* phosphate buffer, pH 7.5. To the clear solution were added 3.5 cc. of salmine solution (= 200 mg. protamine sulfate in 2.5 cc. water plus 1 cc. 0.1 *N* NaOH). The flocculent precipitate was allowed to settle for 2 hr. and then centrifuged off. The supernatant was then dialyzed against running distilled water for 10 hr. Purification: 2980 times; yield: 0.082 mg. protein N; $W = 2800$.

Preparation of Zinc Hydroxide Gel

1280 g. zinc sulfate in 2 l. of water plus 350 g. NaOH dissolved in 2 l. of water. These components are mixed with vigorous stirring and allowed to settle out. The supernatant is removed by suction. The precipitate is washed 3 times with 5 l. of water which is sucked off after settling for ½ hr.

Experiments with Other Adsorbents

Fuller's earth, bentonite silica gel, barium phosphate, copper hydroxide, hematite, and calcium phosphate were also tried, but were not found as satisfactory as the two adsorbents used here, as either complete adsorption or elution of the enzyme presented greater difficulties.

Storage of Uricase Solutions and Preparation of Stored Samples for Injection

The uricase used in most of the experiments was prepared from beef kidney purified through step III. The preparation used showed an O_2 uptake of 2.7 mm.^{3}/min. and a *W* value of 203 after treating the enzyme, dissolved in 0.1 *M* phosphate buffer, with $Al(OH)_3$ gel and centrifuging off the precipitate. The resulting supernatant was placed in cellophane bags (30–40 cc./bag) and stored in a deep freeze unit until needed. To prepare material for injection, the contents of one or more of the bags was allowed to thaw and was centrifuged at 15,000 r.p.m. to obtain a clear brown solution. This solution was then lyophilized and the resulting powder was dissolved in distilled water (35 mg./cc.). No attempt was made to sterilize the preparations. The O_2 uptake of these samples (in the presence of uric acid) ranged from 4.3 to 5 mm.^{3}/min. However, the *W* value was only 114.

The control solutions were made by heating the enzyme powder, dissolved in distilled water, to 100°C. for 15 min. This procedure produced a somewhat milky solution, but there was no deleterious effect following injection of the solution into the chickens. The absence of enzyme activity in the control solutions was established by measuring O_2 uptake of the solution in borate buffer pH 9.6 in the presence of lithium urate at 37°C. No activity was noted.

Determinations of blood uric acid were made on 2 cc. of blood oxalated with lithium oxalate (5). The direct colorimetric method of Folin (6) was used. Protein-free filtrates were prepared by the method of Benedict and Behre (7). All determinations were made in duplicate and the average of the two was used as the level of the blood uric acid.

Fluctuations in the Level of Blood Uric Acid in Normal Chickens

Chickens weighing from 2 to 3.5 kg. were fed 50 g. of ground beef/kg./day. It was found that the blood uric acid level fluctuated widely according to when the chickens were fed. Contrary to the findings of Oppenheimer and Kunkel (1,2) it was impossible to stabilize the blood uric acid level by having meat constantly available, because the chickens would often gorge themselves and then not eat again for 24 hr. or more. Table I illustrates the fluctuations in the blood uric acid level observed in one hen following the consumption of 125 g. of ground beef in 15 min.

TABLE I

The Uric Acid Content of the Blood of a Hen Fed Ground Beef

Exp. conditions	Blood uric acid mg./100 cc.
1 hr. after feeding 125 g. meat	11.8
3 hr. after feeding	8.1
8 hr. after feeding (fasting uric acid level)	6.1

Such fluctuations obviously interfere with the interpretation of results of the injection of uricase solutions. In order to eliminate, as far as possible, the fluctuations following the ingestion of food, the chickens were fed in the late afternoons and bloods were drawn for fasting levels the next morning; the uricase solutions were injected intravenously and a second blood sample was drawn 30 min. later.

Toxicity Experiments

It seemed logical to expect that the intravenous injection of a foreign protein solution would cause anaphylactic reaction following a sensitizing dose of the solution. Three rabbits were injected with 0.5 cc/kg. of the enzyme solution intravenously without noticeable effect. Two weeks later, 2 of the rabbits were given 1 cc. and the third rabbit was given 5 cc. of the enzyme solution intravenously. The rabbit receiving the larger dose became restless almost immediately and 1 min. later developed slow, gasping, stertorous breathing, defecated and urinated, and appeared to be *in extremis*. However, 1 min. later, the respiratory rate increased, the breathing became easier and at the end of an hour the rabbit appeared normal again. The rabbit receiving the smaller doses had milder reactions similar to those observed in the first rabbit, but at no time appeared to be *in extremis.*

During the progress of the experiments several of the chickens received intravenous injections of active or inactivated enzyme preparations at approximately weekly intervals over the course of 6 weeks. Toward the end of this period it was noted that several of the chickens developed an asthmatic type of breathing following injection of the enzyme solution and similar to the reaction noted in the rabbits. This reaction never lasted more than 5 min. and no chicken died of anaphylactic shock.

Experiments to Establish the Effective Dose of Enzyme Solution

Assuming that a dose of 19 mg. of enzyme powder/kg. of chicken would be effective in lowering the blood uric acid (if the total blood volume of a chicken is one-twelfth of its body weight and the plasma volume about one-twenty-fourth of its body weight) the concentration of uricase powder per cc. of plasma following injection and mixing in the blood would be about 0.75 mg./cc. of plasma.

A uric acid solution made up according to the method of Benedict and Hitchcock (8) was added to a sample of pooled human plasma so that the uric acid level of the plasma was 10.1 mg.-%. Two cc. portions of the plasma were then placed in 125 cc. Ehrlenmeyer flasks and various concentrations of enzyme powder in equal amounts of distilled water were added to the flasks. The flasks were then incubated at 37°C. for 1 hr. The proteins were precipitated with 10% sodium tungstate and $N/12$ H_2SO_4 and the filtrates analyzed for uric acid. The results appear in Table II. From the table, it is evident that a concentration of 0.79 mg. of enzyme powder/cc. of plasma almost completely oxidizes the uric acid present in the plasma at the end of one hour's incubation at 37°C. It might be expected that such a concentration of enzyme powder in solution (26 mg./kg. of chicken) would have a demonstrable

TABLE II

Effect of Uricase on Plasma Uric Acid after 1 Hr. Incubation at 38°C.

Sample	Uricase powder added per cc. of plasma *mg.*	Uric acid in plasma *mg.*
1	0	10.2
2	0.079	8.4
3	0.19	5.8
4	0.44	4.3
5	0.79	1.3

effect on the blood uric acid level. However, 5 chickens who received this dosage showed no change in the uric acid level following injections of this concentration of enzyme solution.

Experiments on the Effect of Intravenous Injections of Uricase Solutions on the Blood Uric Acid Level of Chickens

On the basis of the preliminary experiments it was decided to give the chickens a dose of uricase powder of 100 mg./kg. In some cases this amounted to as much as 9 cc. of solution per injection when solutions were made up to contain 35 mg./cc.

TABLE III

Effect of Intravenous Uricase Injections on the Plasma Uric Acid Levels

Uricase, 100 mg. powder/kg. Blood samples were withdrawn before and 30 min. after injection.

Chicken no.	Uric acid in the blood in mg.			
	Active uricase		Inactivated uricase	
	Before	After	Before	After
35	7.4	6.3	—	—
36	5.9	2.3	8.5	7.7
42	4.0	3.0	4.9	5.4
44	8.4	5.9	5.7	5.1
46	8.9	4.7	7.9	6.3
47	4.7	3.5	—	—
34	3.6	3.3	4.0	5.1
39	5.6	4.9	—	—
43	6.5	6.5	—	—

Each chicken served as its own control, being given the active enzyme preparation first and the inactivated preparation a few days later. The results are tabulated in Table III. In all cases except three there was 1 hr. after injection a decrease in the blood uric acid content which ranged from 12 to 61%. The injection of inactivated uricase decreased slightly in some cases the blood uric acid level.

To see whether chickens which showed no response to injection of uricase might have synthesized this enzyme during the course of two months on a meat diet, two of the chickens were sacrificed and their livers tested for enzyme activity. No uricase was found. The possibility was considered that an enzyme inhibitor, perhaps in the nature of an antienzyme, had been produced after repeated injections of enzyme solutions at weekly intervals. The serum of one of the uricase resistant chickens was collected and mixed with equal amounts of an active uricase preparation, water being added to the control instead of serum. No inhibition of enzyme activity was found in the sample to which the chicken serum had been added. There appears to be no adequate explanation of the absence of response to injections of uricase.

The Response to Repeated Injections

In chickens on which the injection of uricase was repeated every two days, the response 1 hr. after injection was always positive, *i.e.*, the blood uric acid was decreased. In no case was the diminution of blood uric acid permanent (Table IV). Some of the chickens which received repeated injections of uricase exhibited mild anaphylactic shock at the time of the injection.

TABLE IV

Effect of Repeated Intravenous Injections of Uricase on Plasma Uric Acid Levels

Uricase, purified to give $W = 940$. Two cc. per injection. Uric acid determined 1 hr. after injections. Uricase injections were given every 2 days.

Chicken no.	Uric acid in the blood	
	Before	After
50		
First injection	8.9	4.8
Second injection	9.0	5.0
Third injection	8.0	5.2
Fourth injection	7.5	4.0

The Duration of the Uricase Effect

The decrease in the blood uric acid levels produced by uricase was of short duration, for the values came back to their pre-injection levels 20 hr. after the injection of uricase. By the rate at which the blood uric

TABLE V

Blood Uric Acid Levels after the Injection of Uricase. Duration of Uricase Action

Uricase purified to give $W = 910$. Two cc. per injection.

Chicken no.	Blood uric acid levels			
	Before	1 hr.	3 hr.	20 hr.
52	11.0	4.0	9.2	10.0
54	9.2	4.6	7.5	11.0
55	8.7	3.9	6.8	9.0

acid rose 3 hr. after injection, it may be predicted that the original values must have been reached before 20 hr. (Table V). Form these experiments it can be concluded that the enzyme loses its activity within 5 hr. after it has reached the blood stream.

SUMMARY

The method presented here for the purification of uricase has given water-soluble enzyme preparations of high activity. The enzyme can be lyophilized and kept at low temperatures for one month with no loss of activity. The enzyme when injected to chickens fed on a meat diet produced a transitory drop in the blood uric acid levels. Repeated injections produced a mild type of anaphylactic shock. From the experiments presented in this paper it may be concluded that the use of repeated injections of uricase to lower blood uric acid levels is not to be recommended.

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The Transformation in the Rat of Carboxyl-labeled Acetate, Methyl-Labeled Acetate, and Labeled Bicarbonate into Amino Acids [1]

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INTRODUCTION

Recent studies have indicated that acetic acid participates in a number of widely diverse metabolic processes. One such paper, by Rittenberg and Bloch (1), has pointed to a relationship between acetate and protein metabolism. The feeding of acetate, labeled in the carboxyl position with C^{13}, to rats and mice, gave rise to C^{13}-containing amino acids in the liver and carcass proteins of these animals. Glutamic and aspartic acids and arginine, isolated from the proteins, and also urinary urea and respiratory carbon dioxide, contained isotopic carbon.

Since there is indication that acetate may be incorporated by way of condensation reactions into intermediates of the tricarboxylic acid cycle (2), and since α-ketoglutaric, oxalacetic, and pyruvic acids are believed to be in biological equilibrium with glutamic acid, aspartic acid, and alanine, respectively, the presence of isotopic carbon in these amino acids following the feeding of acetate might reflect a utilization of acetic acid, without prior degradation to carbon dioxide. On the other hand, experiments such as those of Delluva and Wilson (3) show that carbon dioxide is readily incorporated into carboxyl groups of aspartic and glutamic acids, as well as into the amidine group of arginine.

The present paper compares three C^{14}-labeled compounds: $C^{14}H_3COONa$, $CH_3C^{14}OONa$, and $NaHC^{14}O_3$, with respect to their rates of incorporation into the proteins of various organs of the rat, and their modes of transformation into certain amino acids.

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EXPERIMENTAL

Labeled Compounds

The $CH_3C^{14}OOH$ was synthesized by the method of Dauben, Reid and Yankwich (4), and the $C^{14}H_3COOH$ by that of Tolbert (5).[1a] The former compound had a specific radioactivity of 6.72 μC(microcuries)/mg., and the latter 1.5 μC/mg. Radioactive bicarbonate was prepared by decomposing $BaC^{14}O_3$ (11 μC/mg.) with $HClO_4$, and collecting the evolved $C^{14}O_2$ in a moderate excess of dilute CO_2-free NaOH. The U-type ninhydrin apparatus (6) with the additional feature of a side arm for adding $HClO_4$, was used in this operation.

Administration of Labeled Compounds and Preparation of Proteins from Various Organs of the Rat

Each of three 200 g. male rats received by intraperiotneal injection 32 μC of C^{14} (4,000,000 counts/min. with the Geiger-Müller counter) contained in 20 mg. of one of the following compounds: $CH_3C^{14}OONa$, $C^{14}H_3COONa$, $NaHC^{14}O_3$. Inactive compounds sufficient to make the three doses equivalent were added in the cases of the last two substances.

Each rat was kept in a glass cage for 8 hr. prior to being sacrificed. During this time its urine and expired CO_2 were collected. Protein fractions were prepared from various organs by techniques described in preceding papers (7,8).

Isolation of Radioactive Amino Acids from Proteins of Liver and Intestines

To obtain relatively high concentrations of C^{14} in tissue proteins, labeled acetates, and bicarbonate (in the same amounts specified in the preceding section) were injected in several portions over a 48 hr. period into three 160 g. male rats. The protein powders obtained from the liver (the largest organ) and the intestine (one of the most active organs) of each animal (6 preparations in all) were hydrolyzed with acid and subjected to the carrier method of analysis (9).

The isolated glutamic acid was recrystallized 5 times from hot water, further purified by crystallization from concentrated HCl, and finally crystallized from aqueous solution at pH 3. Aspartic acid was recrystallized 5 times from hot water, purified as the copper salt, and finally recrystallized as the free amino acid. Alanine was recrystallized 3–4 times from concentrated aqueous-alcohol solution, converted to the benzoyl derivative, and the latter recrystallized twice from hot water. Arginine was precipitated and then reprecipitated as the diflavianate, and finally crystallized from concentrated aqueous alcohol solution as the monohydrochloride. Analytical data establishing the purity of amino acids isolated by the foregoing procedures have been given (9).

[1a] Drs. M. Calvin, J. C. Reid and B. M. Tolbert, of the University of California Radiation Laboratory generously supplied the labeled materials for this investigation.

Criteria of Purity in Connection with the Use of Carriers

Analytical determinations, such as carbon and nitrogen analyses, are not sufficiently sensitive to detect the presence of small traces of impurities in re-isolated carriers. Better criteria are: constant specific radioactivity upon repeated recrystallization, and no change in activity upon subsequently transforming the amino acid into a suitable derivative. The carrier technique was particularly suited to the present work, in that the radioactive substances administered were not themselves amino acids, and hence were completely removed in the washing of the proteins with trichloroacetic acid. Of the amino acids which might conceivably be derived from acetate or bicarbonate, glycine, and serine were definitely excluded by the fact that carrier experiments indicated the absence of appreciable C^{14} in these amino acids. The amino acids found to be labeled were the dicarboxylic acids, alanine, and arginine. Table I illustrates the effect of purification on the specific activity of the carriers. In the case of aspartic acid (which is less soluble in water than glutamic acid), a constant specific radioactivity was obtained after 4–5 recrystallizations from hot water. *This specific*

TABLE I

Specific Activity of Amino Acid Carriers at Successive Stages of Purification

The data are from an experiment in which $C^{14}H_3COONa$ was administered to a rat (see Table IV).

Carrier	Purification sequence	Liver protein	Intestinal protein
		counts/min./mg	*counts/min./mg*
Aspartic acid	Recrystallized 5 times from hot water.	6.22	15.0
	Precipitated as copper salt, removed copper, crystallized at pH 3.	5.82	15.8
	Recrystallized from hot water.	6.02	15.0
Glutamic acid	Recrystallized 5 times from hot water.	19.1	29.5
	Crystallized from concentrated HCl, recrystallized at pH 3.	9.14	18.4
	Recrystallized from hot water.	9.78	17.5
Alanine	Recrystallized 4 times from aqueous alcohol.	10.7	21.4
	Prepared benzoyl derivative.	3.84	6.92
	Recrystallized derivative twice from hot water.	4.18	6.40
Arginine	Precipitated as diflavianate, recrystallized latter.	2.28[a]	
	Converted to monohydrochloride.	3.12	

[a] Accuracy of this measurement decreased by particularly large self-absorption correction.

activity was not altered upon further purification by way of the copper salt. Following the repeated recrystallization of glutamic acid from hot water, subsequent crystallization from concentrated HCl produced a marked decrease in specific activity. Thereafter, the specific activity remained constant upon further recrystallization. This behavior indicates that a radioactive contaminant (aspartic acid) was removed by the crystallization from HCl. A similar drop in activity was observed following the benzoylation of alanine, and a constant specific activity of the benzoyl alanine obtained after 2 recrystallizations showed that other labeled amino acids were removed. Precipitation of arginine as the extremely insoluble diflavianate served to free this amino acid from alanine and dicarboxylic amino acids, which form much more soluble diflavianate salts. In addition, the arginine was isolated as the monohydrochloride.

It is significant that the labeled amino acids, isolated with carriers, accounted for an average of 93% of the protein-bound C^{14} in 4 experiments with acetates. Allowing for losses of amino acids during hydrolysis, this value indicates that virtually all of the C^{14} of the proteins was accounted for by the carrier technique.

Degradation of Amino Acids with Ninhydrin

Methods recently described (9) were used to collect the CO_2 evolved from carboxyl groups of alanine, glutamic acid (one COOH), and aspartic acid, and the acetaldehyde[2] derived from alanine. Since the alanine was obtained as the benzoyl derivative, portions of the latter were hydrolyzed with 6 *N* HCl in the autoclave (1 hr. at 15 lb.) prior to treatment with ninhydrin.

Radioactive Measurements

Amino acid and protein samples, generally in the weight range of 20–30 mg., were collected as thin uniform layers on tared aluminum plates of 12.5 cm. area. The samples were counted with the Autoscaler Model Geiger-Müller counter. All values were corrected for self-absortption of radiation by reference to a standard curve, prepared from a series of radioactive glycine samples of varying layer thickness. In correcting $BaCO_3$ samples (derived from decarboxylation of amino acids), the self-absorption data of Yankwich, Norris and Huston (10) were employed. The probable error in counting was less than 3% with all samples, except those with activities less than 1–2 counts/mg./min. These materials of lower acitvity were mainly derived from the administration of $NaHC^{14}O_3$. They include the proteins of muscle, brain, and red blood cells, and certain of the $BaCO_3$ samples. With these materials, the error in counting was approximately 6–7%. The radioactivity measurements were made in duplicate or triplicate on all materials. Average values are recorded in the tables. The degree of reproducibility of measurements was of the order of 4–5%.

In cases in which amino acids were collected as derivatives (benzoyl alanine, acetaldehyde dinitrophenylhydrazone, arginine hydrochloride), the observed activities were recalculated as counts/*mg. of amino acid.*

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[2] Inasmuch as a quantitative recovery of the dinitrophenylhydrazone was difficult to realize, radioactivity calculations were based on the specific activity of the material counted (expressed in terms of an equivalent weight of alanine), rather than on the starting quantity of alanine treated with ninhydrin.

RESULTS AND DISCUSSION

Incorporation of C^{14} into Proteins

Table II compares the activities of the different organs of the rat in the process of C^{14} incorporation into protein, following the administration of $NaHC^{14}O_3$ and labeled acetates. The $C^{14}H_3COONa$ and $CH_3C^{14}OONa$ were utilized to about the same degree, and the relative activities of the organs conformed in both cases to virtually the same pattern as that observed with labeled tyrosine (7), glycine (8), and methionine (11).[3] Since acetate is tranformed into several different amino acids, the pattern, as exemplified in Table II, is most probably a good general indication of the relative protein-synthetic activities of the organs.

TABLE II

Comparison of Acetates and Bicarbonate in the Process of C^{14} Incorporation into Proteins

Organ	Activity as per cent of administered dose/g. protein		
	$C^{14}H_3COONa$	$CH_3C^{14}OONa$	$NaHC^{14}O_3$
Intestinal mucosa	1.75[a]	1.61	0.18
Plasma	0.54	0.48	0.34
Spleen	0.53	0.49	0.065
Lung	0.50	0.48	0.06
Kidney	0.47	0.40	0.045
Liver	0.36	0.37	0.215
Testes	0.22	0.23	0.045
Muscle	0.08	0.07	0.02
Brain	0.06	0.05	0.015
Red blood cells	0.06	0.05	0.00

[a] By way of illustration, this value corresponded to 70 counts/min./mg. protein.

The C^{14} of $NaHC^{14}O_3$ was taken up to a lesser extent than acetate by the proteins of the different organs. It is estimated that 4% of the isotope was incorporated into the total protein of each of the two rats fed labeled acetate, but that only about 1% of the administered $NaHC^{14}O_3$ was incorporated into protein. Also, the pattern for the various organs was somewhat different with isotopic bicarbonate. Thus, with the latter, the intestinal protein was less active than plasma or liver protein.

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[3] Results very similar to those in Table II were obtained when the two acetates were given either intravenously or by stomach tube.

The greater utilization of acetate suggests that it is not transformed extensively into CO_2 prior to incorporation into amino acids. On the other hand, the lesser utilization of labeled bicarbonate can be ascribed, in part, to its more rapid elimination from the body.

Elimination of C^{14}

Table III shows that the elimination of C^{14} by the lungs was virutally complete 8 hr. after the administration of $NaHC^{14}O_3$. The labeled acetates were eliminated to the extent of about 80%. Three to four per cent of the C^{14} was found in the urine in each case.

TABLE III

Degree of C^{14} Elimination 8 Hr. after the Administration of Labeled Acetates and Bicarbonate

Material collected	C^{14} content in per cent of administered dose		
	$C^{14}H_3COONa$	$CH_3C^{14}OONa$	$NaHC^{14}O_3$
Respiratory carbon dioxide	82	80	98
Urine	4.0	2.8	3.7

Distribution of C^{14} Among the Amino Acids of Intestinal and Liver Proteins

The values in Table IV are the percentages of the total C^{14} of each protein contained in its different amino acid components. They cannot indicate the concentrations of isotope in the amino acids unless the exact proportions of the latter in the proteins are determined by a separate analytical method.

Four amino acids: glutamic acid, aspartic acid, alanine, and arginine, accounted for nearly all of the C^{14} in the proteins. The lowest recovery was that of intestinal protein following the administration of $NaHC^{14}O_3$ in which only $\frac{2}{3}$ of the C^{14} was accounted for. Also, low C^{14} concentrations were found in glycine and serine isolated with the aid of carriers.[4]

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[4] The glycine and serine, after repeated recrystallization, each accounted for approximately 2% of the total C^{14} in the different proteins. In view of these low radioactivities, it was not feasible to prepare derivatives of the two amino acids.

TABLE IV

Distribution of C^{14} Among Amino Acids of Intestinal and Liver Proteins after the Administration of Labeled Acetates and Bicarbonate to Rats

Eight parts of carrier per part of (hydrolyzed) protein were employed in the case of intestinal proteins, and 4 parts of carrier with liver proteins, in the isolation of amino acids. The percentage of the total C^{14} in a protein due to an individual amino acid, A, was calculated (9) by the formula: $\frac{\text{specific activity of } A \text{ (diluted with carrier)}}{\text{specific activity of protein}} \times \frac{\text{weight of } A}{\text{weight of protein}} \times 100.$

Amino acid isolated	$NaHC^{14}O_3$ administered				$C^{14}H_3COONa$ administered				$CH_3C^{14}OONa$ administered			
	Intestinal protein		Liver protein		Intestinal protein		Liver protein		Intestinal protein		Liver protein	
	Counts min./mg	% of total C^{14}	Counts min./mg.	% of total C^{14}	Counts min./mg	% of total C^{14}	Counts min./mg	% of total C^{14}	Counts min./mg	% of total C^{14}	Counts min./mg.	% of total C^{14}
Original protein	72.0	100	33.0	100	326	100	108	100	308	100	77.5	100
Reactive COOH groups in hydrolyzed protein	47.0	65.3	18.9	57.4	59.8	18.4	19.0	17.6	157	51.0	38.6	49.8
Glutamic acid	2.20	24.5	1.70	20.6	17.5	42.9	9.78	36.2	12.6	32.8	8.1	41.8
Aspartic acid	1.92	21.3	2.04	24.7	15.0	36.8	6.02	22.4	12.4	32.3	3.94	20.3
Alanine	1.78	19.8	1.52	18.4	6.4	15.7	4.18	15.5	9.3	24.2	3.96	20.4
Arginine	0.22	2.4	2.36	28.6	0.48	1.2	3.12	12.6	0.42	1.0	3.16	16.3
Sum of isolated amino acids		68.0		92.3		96.6		86.7		90.3		98.8
α-COOH of glutamic acid	1.82	20.2	1.70	20.6	3.32	8.1	1.68	6.2	5.28	13.7	3.50	18.1
Both COOH's of aspartic acid	1.64	18.2	1.98	24.0	4.78	11.7	1.70	6.3	10.58	24.9	4.16	21.5
COOH of alanine	1.42	15.8	1.22	14.8	1.16	2.85	0.92	3.4	7.28	18.9	3.72	19.2
$\alpha + \beta$ carbons of alanine (acetaldehyde)	[a]		[a]		4.80	11.8	2.88	10.7	[a]		[a]	

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[a] The radioactivity was too low for satisfactory measurement.

In the three intestinal protein preparations, the C^{14} was contained chiefly in glutamic acid, aspartic acid, and alanine. In the liver proteins, arginine had a moderate concentration of C^{14} following the administration of acetates, and a relatively high C^{14} concentration following $NaHC^{14}O_3$. Reference has already been made to the similar findings of previous investigators.

CO_2 Fixation in Relation to Acetate Metabolism

The extent of CO_2 fixation following the administration of labeled acetates could be gauged by comparing the degree of C^{14} incorporation into COOH groups of amino acids, with the corresponding values obtained after giving $NaHC^{14}O_3$. The C^{14} concentration in amidine groups of liver arginine gave an added indication of CO_2 fixation.

Table IV shows that, following the administration of $NaHC^{14}O_3$, the CO_2 evolved from COOH groups of hydrolyzed proteins with ninhydrin represented 57 and 65% of the total isotope in liver and intestinal proteins, respectively. Totaling the individual values for carboxyl-C^{14} of glutamic acid, aspartic acid, and alanine, gave 59% for liver protein. Taking into account the high C^{14} concentration in liver arginine, CO_2 fixation accounted for 87% of the total C^{14} in liver protein.[5]

After the feeding of $C^{14}H_3COONa$, only 18% of the total C^{14} in hydrolyzates of either intestinal or liver protein was liberated by ninhydrin. Comparable results, 23 and 16% are obtained by totaling the COOH-C^{14} values for glutamic acid, aspartic acid, and alanine. Taking into account the C^{14} of arginine, and assuming that the γ-COOH of glutamic acid contains approximately the same C^{14} concentration as the α-COOH, the fixation of CO_2 accounts for about 33 per cent of the C^{14} in either liver or intestinal protein. This value is less than half as large as that obtained with liver protein following $NaHC^{14}O_3$ administration.

Accordingly, a sizeable portion, probably more than half, of the acetate appears to have been transformed into amino acids without prior breakdown to CO_2. The same conclusion is reached by a direct comparison of the liver arginine values, following the administration of $NaHC^{14}O_3$ and $C^{14}H_3COONa$.

Examination of the results with $CH_3C^{14}OONa$ indicates that, as in the $NaHC^{14}O_3$ experiment, almost all of the C^{14} was present in COOH and amidine groups of amino acids. The COOH groups in hydrolyzates accounted for 51 and 50% of the total C^{14} in intestinal and liver proteins, respectively. Noticeably higher values, 62 and 59%, are obtained by totaling the COOH values for dicarboxylic amino acids and alanine. Estimating the γ-COOH of glutamic acid as before, and adding the C^{14} of arginine, it is seen that more than 95% of the C^{14} of liver protein was present in COOH plus amidine groups. The corresponding value for intestinal protein is approximately 85%. The extent of breakdown of $CH_3C^{14}OOH$ to CO_2 cannot be gauged from these results. It may be noted, however, that the liver arginine value is somewhat higher than that with $C^{14}H_3COOH$, but still much lower than that obtained with $NaHC^{14}O_3$.

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[5] A similar calculation is not given for intestinal protein, in view of the fact that only 68% of the C^{14} was accounted for by isolated amino acids.

Mechanisms of Carbon Dioxide and Acetate Metabolism

An insight into the metabolic paths by which CO_2 and acetate are incorporated into amino acids is obtained by examination of the individual decarboxylation experiments with glutamic acid, aspartic acid, and alanine, recorded in the lower portion of Table IV. A comparison of these results with those in the upper part of the table reveals the fraction of the C^{14} in each amino acid that is present in COOH groups (reactive to ninhydrin).

Thus, it is seen that, following the administration of $NaHC^{14}O_3$, the aspartic acid of liver protein contained 24.0/24.7 × 100 or 97%, and the aspartic acid of intestinal protein 18.2/21.3 × 100 or 86%, of the C^{14} in COOH groups. The COOH of alanine from either liver or intestinal protein contained 80% of the C^{14} in the alanine molecule. All of the C^{14} of liver glutamic acid, and 83% of that in intestinal glutamate, was in the one COOH α to the amino group.

The foregoing results, particularly those pertaining to liver protein, are in agreement with the work of Wood, Werkman, Hemingway and Nier (12), who studied the metabolism of $C^{13}O_2$ in pigeon liver. The $C^{13}O_2$ gave rise to carboxyl-labeled lactate, C_4-dicarboxylic acids labeled exclusively in COOH groups, and α-ketoglutarate labeled only in the COOH α to the carbonyl group. To explain these results, Wood and coworkers postulated a reversible reaction between pyruvate and labeled CO_2, yielding COOH-labeled enol-oxalacetate, together with COOH-labeled pyruvate (by the reverse reaction). The participation of the oxalacetate in the tricarboxylic acid cycle would lead to ketoglutarate labeled exclusively in the α-COOH position only if an unsymmetrical compound, such as isocitric acid, were a precursor of the ketoglutarate.

With reference to the transformation of labeled acetate into the intermediates of the tricarboxylic acid cycle, a plausible mechanism appears to be a condensation of acetate and oxalacetate to yield *cis*-aconitate (2). If it is again assumed that citric acid lies outside the direct path of metabolism, $C^{14}H_3COOH$ should yield ketoglutarate, oxalacetate, and pyruvate labeled only in non-COOH positions. This same conclusion holds if an "end to end" condensation of pyruvate and $C^{14}H_3COOH$ to yield ketoglutarate, or of two $C^{14}H_3COOH$ molecules to oxalacetate, is assumed (2).

The results with $C^{14}H_3COONa$ in Table IV are in agreement with the acetate-oxalacetate condensation theory if the minor fractions of carboxyl-C^{14} in the amino acids are ascribed to CO_2 fixation.

The condensation of $CH_3C^{14}OOH$ with oxalacetate should give rise to carboxyl-labeled alanine and aspartic acid, and the glutamic acid labeled only in the γ-COOH position. The expectations relative to alanine and aspartic acid are fairly well fulfilled by the data in Table IV. But the glutamic acid from both liver and intestinal protein had approximately 40% of its label in the α-COOH group. In this connection, it may be noted that Rittenberg and Bloch (1) found about 25% of the isotopic carbon to be present in the α-COOH of liver glutamic acid, following the feeding of $CH_3C^{13}OOH$ to rats. Weinhouse, Medes and Floyd (13) incubated carbonyl- and carboxyl-labeled acetoacetate with rat kidney homogenate, and isolated from the mixture α-ketoglutarate which contained $\frac{1}{4}$ to $\frac{1}{6}$ of its isotope in the α-COOH position.

To reconcile the glutamic acid results obtained by us and by other workers with the postulate that an unsymmetrical tricarboxylic acid is a precursor of α-ketoglutarate, it is necessary to assume that a considerable fraction of the isotopic carbon of glutamic acid was derived from CO_2 fixation. In the light of the data presented in this paper, it seems reasonable to assume that the glutamate derived approximately 60% of its C^{14} directly from labeled acetate, and the remainder from $C^{14}O_2$, resulting from breakdown of the acetate.

SUMMARY

The three labeled compounds, $NaHC^{14}O_3$, $CH_3C^{14}OONa$, and $C^{14}H_3COONa$, were compared with respect to their rates of incorpora-

tion into the proteins of various organs of the rat, and their transformation into alanine, aspartic acid, glutamic acid, and arginine.

The C^{14} of the two labeled acetates was incorporated to about the same extent, while the bicarbonate gave rise to considerably lower C^{14} concentrations, in the tissue proteins.

When $C^{14}H_3COONa$ was administered, only a minor fraction (less than $\frac{1}{3}$) of the C^{14} in either liver or intestinal proteins was present in COOH plus amidine groups of amino acids. When either $CH_3C^{14}OONa$ or $NaHC^{14}O_3$ was fed, almost all of the C^{14} was found in these groups. The lower concentrations in the $C^{14}H_3COONa$ experiment indicate that much of the acetate was transformed into amino acids by condensation reactions, without prior degradation to CO_2.

The positions of the C^{14} in the molecules of glutamic acid, aspartic acid, and alanine, isolated from liver and intestinal proteins, were examined in relation to the theory that acetate enters the tricarboxylic acid cycle by condensation with oxalacetate. The experimental data, in the main, supported this theory.

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Effect of Vitamin P Compounds on the Action of Histidine Decarboxylase

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INTRODUCTION

Certain flavonoid compounds have been shown to have the property of decreasing capillary permeability and fragility (1). The group of these compounds has been designated by the term "vitamin P." The mechanism of action of these substances is unknown, although the theory has been advanced that they inhibit the destruction of epinephrine in the body (2). It has also been suggested that vitamin P substances function through an inhibition of the action of hyaluronidase (3).

Swyer (4) has reported that the inhibition of skin diffusion by salicylate is due to an anti-histamine action rather than to an inhibition of hyaluronidase, while Mayer and Kull (5) have shown that hyaluronidase is inhibited *in vivo* by anti-histamines. These findings, pointing to some relationship between hyaluronidase and histamine, as well as the known influence of histamine on capillary permeability, suggested the utility of investigating the effect of vitamin P substances on histidine decarboxylase, the enzyme which is presumably responsible for the formation of histamine in the animal body. Point was lent to this by the fact that the vitamin P compounds are rich in hydroxyls, and it has been shown (6, 7) that substances with phenolic hydroxyl groups inhibit the action of histidine decarboxylase.

EXPERIMENTAL

The method used for this investigation was a modification of that of Werle (8), as previously reported (7). The vitamin P compounds used were chosen to provide a variety of different structures within the flavonoid group. Where aglycones were compared with compounds containing a sugar residue the weight of the latter used was doubled to allow for the increase in molecular weight due to the sugar. The hesperidin derivatives tested had been previously prepared and shown to be effective inhibitors of hyaluronidase (9). Ascorbic acid was tested because it too had been shown to be a hyaluronidase inhibitor (3). Catechin red, the so-called "anti-vitamin P," was prepared by the method of Etti (10).

RESULTS

Table I gives the action of the substances tested on histidine decarboxylase.

TABLE I

Action of Flavonoid Compounds on Histidine Decarboxylase

Compound	Inhibition (per cent)		
	0.1 mg./cc.	1.0 mg./cc.	2.0 mg./cc.
Ascorbic acid	—	15	—
Quercetin	0	100	—
Rutin	—	0	0
Hesperidin	—	0	—
Homoeriodictyol	—	30	—
Phosphorylated hesperidin	—	0	0
Sulfonated hesperidin	—	0	0
Hesperidin methyl chalcone	—	0	0
Esculin	—	0	0
Esculetin	—	30	—
d-Catechin	60	100	—
Catechin red	—	0	100

It will be noted that, in every case, inhibition was caused by aglycone flavonoids and not by the corresponding compounds containing a sugar residue. In the case of hesperidin, solubility was not complete at the concentration noted, and some of the compound precipitated out during the course of the experiment.

Some of the compounds which had been shown to be inactive themselves were tested in combination with ascorbic acid, to ascertain whether they would have any effect in potentiating the action of the ascorbic acid. These results are presented in Table II. Because of its insolubility, hesperidin was not tested in this study. In each case the concentration of the compounds was 1.0 mg./cc.

TABLE II

Action on Histidine Decarboxylase of Combinations of Flavonoids and Ascorbic Acid

Combination	Inhibition (per cent)
Ascorbic acid + rutin	50
Ascorbic acid + hesperidin methyl chalcone	50
Ascorbic acid + phosphorylated hesperidin	0
Ascorbic acid + esculin	0

Discussion

The results indicate that the action of histidine decarboxylase is inhibited by vitamin P compounds. The mechanism of the inhibition is unknown, although the work of Bartlett (11) appears to be pertinent. Bartlett suggests that the activity of the flavonoids is due to the formation of quinones, which then react either with the sulfhydryl or the amino groups of proteins. Some support for this view is afforded by the fact that *p*-benzoquinone has been shown to be an effective inhibitor of histidine decarboxylase (7). Since the inhibitors act only in concentrations of 1:1000 to 1:10,000, the inhibition is probably nonspecific.

Why a sugar-containing flavonoid should be inactive, while its aglycone is active, is doubtful. It is possible that the activity is destroyed by the blocking of the hydroxyl group to which the sugar is attached; it seems more likely, however, that the sugar residue affords some sort of steric hindrance to the action of the compound.

The reason for the potentiation of the action of ascorbic acid noted is again not known. It is suggestive, however, that the compounds tested which did not show this action were incapable of quinone formation, esculin having only one free hydroxyl group and phosphorylated hesperidin none in the ring system.

d-Catechin was by far the most effective of the compounds tested; the action of catechin red, its anhydride, is apparently toxic, changing from zero to complete inhibition over a relatively small range of concentration. No "anti-vitamin P" effect, however, was observed with the catechin red.

While the mechanism of the action of these vitamin P compounds on histidine decarboxylase is far from clear, the fact that they do act on a system which may play a role in the maintenance of capillary strength is suggestive. It leads to the idea that the physiological action of "vitamin P" may not be a specific one, but may be exerted on several different systems all of which play a role in the maintenance of normal capillary function.

SUMMARY

Several flavonoid compounds have been shown to inhibit the action of histidine decarboxylase *in vitro*.

Certain of the compounds tested have been shown to potentiate the inhibitory action of ascorbic acid on the enzyme.

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A Spectrophotometric Method for the Determination of the Protein Content of Mushroom Tyrosinase Preparations

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INTRODUCTION

Most of the tyrosinase preparations studied in these laboratories have been prepared from the cultivated mushroom, *Psalliota campestris* (1 5). The fact that the mushroom enzyme is quite unstable in the original aqueous extract, and becomes more stable only after partial removal of accompanying substrate and foreign protein material, makes it generally advisable to carry out several stages of the purification process within the shortest possible time.

Previous to the present investigation no rapid method was available for the determination of the protein content of tyrosinase preparations at successive stages in the purification process. The degree of purification was estimated at each stage by correlating the activity with dry weight determinations made on the undialyzable solids, and delays of 2–4 days for dialysis were unavoidable. To minimize losses in total activity, it was frequently necessary to carry out several stages of the purification without concurrent knowledge of the degree of purification achieved at each stage, for only activity measurements could be made in the time allowed.

The method proposed below, based on a spectrophotometric determination of the total protein content, corrected for pigment content, has the marked advantage of being rapid and applicable concurrently with the activity measurements. Of equal advantage, is the fact that it is not necessary in the proposed spectrophotometric method to sacrifice a portion of the enzyme in order to determine its protein content. This point is of great significance during the later stages of the purification process where only a relatively few milligrams of total protein material may be involved.

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METHOD

It has long been known that colorless proteins exhibit selective absorption in the ultraviolet region of the spectrum with a maximum absorption at a wavelength of about 280 mμ and no significant absorption at wavelengths greater than 325 mμ (6, 7). This characteristic absorption band of proteins has been interpreted as being caused by the aromatic amino acid components, tryptophan and tyrosine (6). Some of the absorption in the same region is also attributed to the presence of the peptide linkages, for gelatin, which contains no tyrosine or tryptophan, shows some selective absorption in the 280 mμ region (8). The intensity of the latter, however, is much lower than that due to the aromatic amino acids. Holiday (6) compared the absorption curves of various proteins with the absorption curves of tyrosine and tryptophan mixtures made up so that the concentrations of these amino acids corresponded to those found in the protein. He found that the summation curves approximated very closely those of the proteins at a wavelength of 275 mμ and at longer wavelengths. The agreement was within the experimental error (±0.05 *E*) except in cases where there was much pigment. On the basis of these results, it would seem that a determination of the extinction[2] ($E = \log I_0/I$) at 280 mμ might be used to calculate the concentration of protein in solutions where the extinction coefficient of that protein is known.

Green and associates (10) made use of this principle during the isolation and purification of zymohexase. They used the ratio

$$\frac{\log I_0/I \text{ at } 280\ \text{m}\mu}{\text{enzyme units/ml.}}$$

as a measure of the purity level of the enzyme in successive stages of its purification. They gave no data but stated that the factor for converting the density values ($\log I_0/I$) into dry weight/mg. was remarkably constant throughout the entire purification process.

When an attempt was made to apply this principle to the purification of mushroom tyrosinase, it soon became apparent that, in the early stages of purification, where tyrosinase samples are highly colored, a considerable part of the absorption in the 280 mμ and adjacent regions of the spectrum was due to accompanying nonprotein pigment material (see Fig. 1). The absorption curves of Fig. 1 reveal that the typical protein absorption maximum at 280 mμ developed only after several stages in the purification, during which much of the colored material was removed. The very early samples (see Curve 1) exhibited a maximum absorption at about 260 mμ, presumably due to contaminating phenolic substrate materials. This phenol maximum was

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[2] The term *density* is also used by some authors for the value of $\log I_0/I$. The terms *extinction* (*E*), *transmission* (*T*), *absorption* (*A*), and *extinction coefficient* (*k*) are used in this communication as defined by Brode (9).

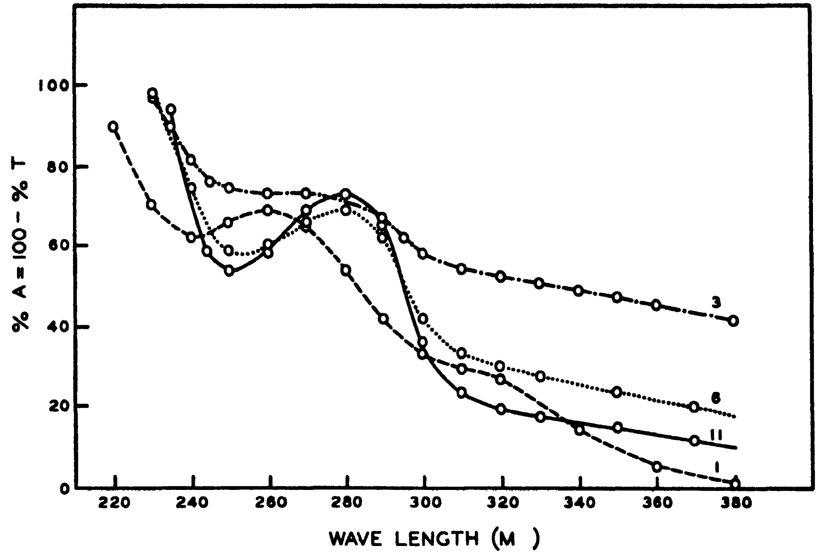

FIG. 1. Showing the development of the protein absorption maximum at 280 mμ during successive stages of purification of mushroom tyrosinase. The curve numbers correspond to the purification stages outlined in the text.

practically gone, however, by the third stage in the purification (see Curve 3) but a considerable absorption of nonselective and nearly linear nature remained in the regions of wavelengths longer than about 320 mμ (presumably due to pigment). With each succeeding stage in the purification the absorption at the longer wavelengths (greater than 320 mμ) showed a progressive decrease (see Curves 6 and 11) corresponding to loss in color. No change in the absorption curves of the tyrosinase samples was observed after they had been allowed to stand at refrigerator temperature for several days. In other words, the amount and nature of the pigment contribution to the total absorption did not change with time.

At first, an attempt was made to evaluate the pigment absorption at 280 mμ by deproteinizing the colored enzyme samples with trichloroacetic acid and measuring the extinction and dry weight of the protein-free pigment material. The results of several such experiments revealed, however, that a considerable and variable part of the pigment was always precipitated with the protein, and the remaining pigment could not be obtained in reproducible form. The ratio of the extinction at 280 mμ to the dry weight of the deproteinized sample (the extinction coefficient) varied from one sample to another. The results obtained with 3 deproteinized samples, prepared in the same way from specimens of the colored enzyme at 3 different stages in the purification are shown in Table I. In these 3 cases the average deviation from the mean ($k = 9.17$) was ±13.6%. The ultraviolet absorption curves of the same 3 deproteinized samples are shown in Fig. 2.

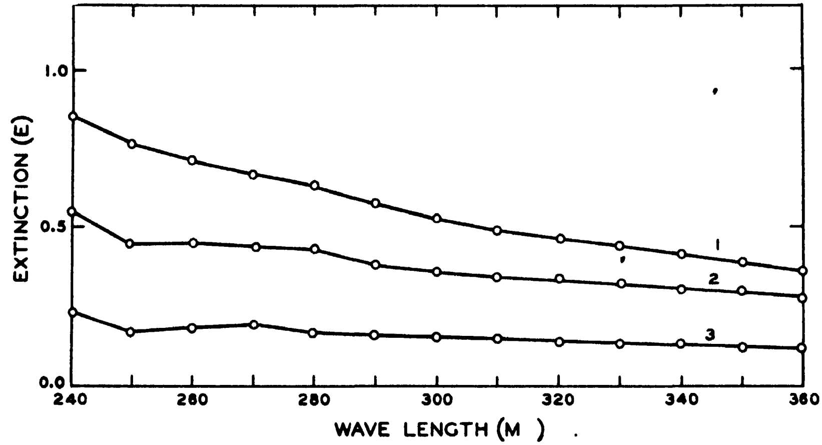

FIG. 2. Showing the absorption curves of 3 deproteinized tyrosinase samples. Note the nonselective and nearly linear character of the absorption in each case from wavelengths of about 300 mμ to 350 mμ. See legend of Table I for method of preparing the deproteinized samples.

It can be seen from the curves of Fig. 2 that the absorption of the pigment material between 300 mμ and 360 mμ in each case was nonselective and practically linear in character. The slight increase in absorption in the 280 mμ region (about a 10% departure from linearity in the cases of Samples 1 and 2) was probably due to small amounts of protein or protein fragments remaining in these samples after the trichloroacetic acid treatment. The presence of small and variable amounts of such protein material in the samples could easily account for the variation in the extinction coefficients at 280 mμ recorded in Table I, particularly if the extinction coefficient of such protein material was relatively low in comparison with that of the pigment. In other words, a slight increase in absorption at 280 mμ could correspond to a significant amount of protein dry weight matter.

TABLE I

Showing the Variation in the Extinction Coefficient at 280 mμ of Three Deproteinized Tyrosinase Pigment Samples

Deproteinized sample	Log I°/I, 280 mμ. E observed	Conc. (C) mg./cc.	Extinct. coeff. $k = E/C$	Deviation from mean
1	0.63	0.057	11.05	+1.88
2	0.43	0.054	7.96	−1.21
3	0.17	0.020	8.50	−0.67
			Mean: 9.17 a.d. = ±1.25 (±13.6%)	

The colored enzyme samples were deproteinized at room temperature by treatment with an equal volume of 10% trichloroacetic acid. After standing 30 min., the precipitate (usually colored) was removed by centrifugation and the colored filtrate was dialyzed for 60–70 hr. against distilled water to remove salts, trichloroacetic acid and low molecular weight protein fragments. The pigment material did not diffuse through the membrane or precipitate during the dialysis. The ultraviolet absorption of the pigment solutions was measured (see Fig. 2), and the concentration of pigment material was estimated by evaporation to dryness at 104–5°C.

Except for enzyme samples 1 and 2 in the purification procedure, the nonselective and practically linear nature of the absorption observed in the 320–380 mμ region for all enzyme samples (see Fig. 1) was strikingly similar to that of the deproteinized samples shown in Fig. 2. This observation suggested that the absorption in this region was primarily due to the pigment material associated with the enzyme protein. Furthermore, it was apparent that the protein maximum at 280 mμ developed during the purification procedure, and the amount of absorption in the 320–380 mμ region, attributed to pigment, continuously decreased without changing its nonselective and practically linear character. These observations suggested that an evaluation of the pigment extinction ($E_{\text{pig.}}$) at 280 mμ for all except the first two enzyme samples might be made by extrapolating the nonselective pigment absorption into the 280 mμ region (see Fig. 3). By subtracting the extrapolated value of $E_{\text{pig.}}$ from the observed extinction ($E_{\text{obs.}}$) at 280 mμ, the extinction due to protein ($E_{\text{prot.}}$) at that wavelength would be obtained.

It is apparent that the method of extrapolation suggested above for obtaining a corrected value of $E_{\text{prot.}}$ at 280 mμ is based on two assumptions:

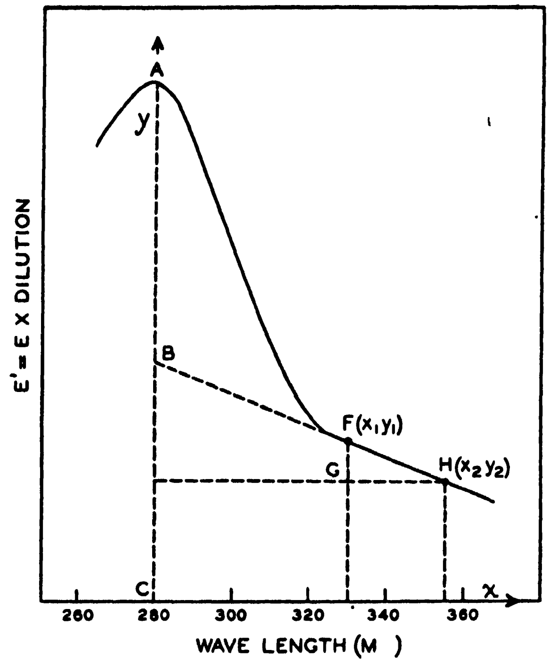

FIG. 3. Illustrating the method of extrapolation for determining the value of $E'_{\text{prot.}}$ at 280 mμ.

$$\text{Eq. 1.} \quad E'_{\text{prot.}} = E'_{\text{obs.}} - E'_{\text{pig.}} = AC - BC.$$

Taking AC as the ordinate and CD as the abscissa and representing the absorption curve of the pigment by the line BFH passing through points $F(x_1, y_1)$ and $H(x_2, y_2)$, where

$$x_1 = 330 - 280 = 50 \text{ m}\mu, \; x_2 = 355 - 280 = 75 \text{ m}\mu.$$
$$y_1 = E'_{330 \text{ m}\mu}, \qquad y_2 = E'_{355 \text{ m}\mu},$$

the general equation of the straight line can be used:

Eq. 2: $$y = mx + b.$$

Since the slope (m) of this line

$$= -FG/GH = -(E'_{330 \text{ m}\mu} - E'_{355 \text{ m}\mu})/25$$

and $$b = E'_{\text{pig., } 280 \text{ m}\mu}.$$

Eq. 2 becomes:

Eq. 3: $$y = \frac{E'_{355 \text{ m}\mu} - E'_{330 \text{ m}\mu}}{25}\, x + E'_{\text{pig., } 280 \text{ m}\mu}.$$

Substituting the values x_1 and y_1 in Eq. 3, and rearranging the equation to solve it for $E'_{\text{pig., } 280 \text{ m}\mu}$:

1. that the absorption curve due to pigment is linear throughout the region 280–360 mμ, and
2. that the extinction coefficient of the protein matter does not change significantly during the purification procedure. The latter assumption is equivalent to assuming that the tyrosine and tryptophan compositions of the enzyme protein and of the non-enzymatic protein impurities are essentially the same.

Since nothing was known about the tyrosine and tryptophan composition of mushroom tyrosinase protein matter, the simplest experimental method of testing the validity of the above assumptions was to determine the constancy of the protein extinction coefficient ($k = E/C$) throughout the preparative procedure, using the values of $E_{\text{prot.}}$ at 280 mμ as obtained by the method of extrapolation and correlating with the protein concentrations as measured by the dry weight or the nitrogen content of the undialyzable material.

It is the purpose of this communication to report that the above experimental method leads to a satisfactorily constant value of the protein extinction coefficient throughout successive stages of the enzyme purification. One may, therefore, conclude that the above assumptions, on which the method of extrapolation is based, are valid within the limits of experimental error.

EXPERIMENTAL

The procedures described below were developed using two tyrosinase preparations (C259 and C260) made from the common cultivated mushroom (*Psalliota campestris*). Both preparations were purified only to the extent of establishing whether or not a constant relationship existed between the corrected protein extinction at 280 mμ and the protein content as determined by the dry weight and nitrogen content measurements. The purification stages at which samples were taken are given in outline form below. The activity data, sample volumes, dry weight, *etc.*, are given in Table II.

Eq. 4:

$$E'_{\text{pig., 280 m}\mu} = E'_{\text{330 m}\mu} - \frac{E'_{\text{355 m}\mu} - E'_{\text{330 m}\mu}}{25}(50),$$

$$E'_{\text{pig., 280 m}\mu} = 3E'_{\text{330 m}\mu} - 2E'_{\text{355 m}\mu}.$$

Combining Eq. 1, and Eq. 4:

Eq. 5:

$$E'_{\text{prot., 280 m}\mu} = E'_{\text{obs., 280 m}\mu} - 3E'_{\text{330 m}\mu} + 2E'_{\text{355 m}\mu}.$$

TABLE II

Enzyme prepn. number	Purification stage	Total volume cc.	Dry wt. mg./cc.	Activity units/mg		E'prot.	E'prot./mg./cc.	Mean
				Cat.	Cres.			
	1	19000	2.23	148	14.3	--	—	
	2	5200	3.85	304	34.3	—	--	
	3	2000	5.97	497	37.7	—	—	
	4	2200	—	—	—	—	—	
	5	2400	3.68	343	19.0	4.88	1.32	
C259	6	2400	1.00	525	14.5	1.31	1.31	
	7	210	5.67	1410	--	7.42	1.31	
	8	260	4.62	1260	16.2	6.90	1.49	1.37
	9	33	10.30	311	17.0	13.83	1.34	±0.09
	10	55	7.02	1080	32.0	10.52	1.50	(a.d.)
	11	60	6.75	2445	37.0	8.81	1.31	
	1	15000	1.92	83	39.0	—	—	
	2	5000	3.14	114	20.7	—	—	
	3	2100	4.07	182	--	5.40	1.33	
	4	2200	—	—	—	—	—	
	5	2300	2.01	189	—	2.59	1.29	
C260	6	2300	1.02	255	—	1.53	1.50	
	7	230	5.00	507	27.6	7.73	1.54	1.45
	8	265	3.75	582	32.0	5.43	1.45	±0.09
	9	77	6.40	282	7.0	9.90	1.54	(a.d.)
	10	25	3.64	1155	34.4	5.28	1.45	
	11	65	5.37	932	28.0	7.94	1.48	
	4x		9.32	77	10.2			
	6x		3.55	84	14.7			

Mean for both series = 1.41 ± 0.09 (a.d.) = ±6.9%

The method of calculating the values of $E'_{\text{prot.}}$ is illustrated below, using the data obtained on sample C259-6, where $E' = (\log T_0/T) \times$ dilution.

	%T	log %T	log To/T	Dil.	E'	$3E'$ 330mμ	$2E'$ 355mμ
280	60.2	1.7796	0.2206	1:8	1.76		
330	47.8	1.6794	0.3206	1:1	0.32	0.96	
355	55.9	1.7474	0.2526	1:1	0.25		0.51

And $E'_{\text{prot.}} = E'_{280\ m\mu} - 3E'_{330\ m\mu} + 2E'_{355\ m\mu} = 1.31.$

Enzyme Purification

Stage 1. Fresh mushrooms (30 lb.) were minced and washed several times with dry-ice cooled acetone. After filtration and pressing, the dry cake was frozen on dry ice. The suspension obtained on homogenizing the cake in 4 volumes of water was allowed to stand overnight and then filtered. (Sample 1 = dark brown solution.)

Stage 2. Protein precipitated with 1.5 volumes of acetone, filtered,[3] and redissolved in 5 l. of cold water. (Sample 2 = dark brown solution.)

Stage 3. Solid $(NH_4)_2SO_4$ added to 0.6 saturation and precipitated crude enzyme washed with 0.6 saturated $(NH_4)_2SO_4$ solution. Precipitate dissolved in 2 l. of water. (Sample 3 = brown solution.)

Stage 4. Above solution mixed with 0.1 volume of alumina gel, prepared according to Willstätter (11) and filtered Filtrate kept for further purification (see Stage 5) while the solids obtained at this stage in both preparations C259 and C260 were combined and eluted from the filter pad and gel with 0.2 *M* Na_2HPO_4 and filtered. (Sample 4X = very dark solution.) Because of the low order of purity of this sample (see Table II) no further purification was attempted.

Stage 5. Filtrate from alumina treatment (stage 4) dialyzed against running cold water overnight. (Sample 5 = brown solution.)

State 6. Lead subacetate solution[4] (0.1 saturated) added to above dialyzate unti an appreciable precipitate formed. Filtration gave a lightly colored solution (sample 6). The residues at this stage in both preparations C259 and C260 were combined and eluted with 0.2 *M* Na_2HPO_4. Filtration gave a highly colored solution (sample 6X) of low purity (see Table II) which was not further purified.

Stage 7. Filtrate (sample 6) again treated with 0.1 volume of alumina gel, filtered, eluted with a small volume of 0.2 *M* Na_2HPO_4 and again filtered. (Sample 7 = brown solution.)

Stage 8. Salts removed by dialysis against running tap water. (Sample 8.)

Stage 9. A fraction of the enzyme precipitated by adding 0.1 volume of cold acetone followed by enough 0.1 saturated lead subacetate solution to produce cloudiness. Precipitate filtered off, suspended in 0.2 *M* Na_2HPO_4 and refiltered. (Sample 9 = dark brown solution.)

Stage 10. A second fraction of the enzyme precipitated by addition of more lead subacetate to first filtrate of stage 9. Precipitate treated in the same way as that above. (Sample 10 = lighter colored solution.)

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[3] All suction filtrations throughout the course of preparation were carried out using a quarter-inch pad of Celite (Johns Manville, No. 535) on the filter paper in the Buchner funnel. In most cases, the precipitated protein was separated from the Celite by dissolving in water and refiltering. After alumina absorptions the precipitates were eluted from the pad and alumina using 0.2 *M* Na_2HPO_4 solution.

[4] The lead subacetate solution was prepared as follows: 420 g. of Pb $(C_2H_3O_2)_2 \cdot 3H_2O$ and 140 g. of PbO were ground in a mortar and mixed with 1400 cc. of water in a glass-stoppered bottle. After thorough shaking it was allowed to stand for a week at room temperature and then filtered. One volume of the filtrate was diluted to 10 volumes with water to make 0.1 saturated lead subacetate solution.

Stage 11. Filtrate from stage 10 lead subacetate treatment mixed with 0.05 volume of alumina gel and filtered. Adsorbate eluted with 0.2 *M* Na_2HPO_4 and again filtered. (Sample 11 = clear brown solution.)

Measurement of Protein Concentration and Enzyme Activity

On each of the enzyme samples mentioned above 3 measurements were made: enzyme activity, ultraviolet absorption, and dry weight. On certain of the samples nitrogen was also determined. The activity measurements (both catecholase and cresolase activity[5]) were made on the original sample which was then centrifuged if necessary and filtered through a sintered glass filter. The optically clear sample thus obtained was then used for all the other determinations. In all cases the enzyme activities were checked after clarification, and in no case was there a significant difference in activity observed. Catecholase activity was measured by the chronometric method (12). Cresolase activity was measured by the method of Gregg and Nelson (13). All light-absorption measurements were made using a Beckman Model DU spectrophotometer with ultraviolet attachment employing 1 cm. quartz cells. Dry weight determinations were made in duplicate as described by Mallette (14). Nitrogen determinations were made on certain of the dry weight residues using the micro Dumas method.

In the spectrophotometric measurements the optically clarified samples were diluted so as to give readings between 20 and 60% transmission. Using water as a blank, readings were always recorded at 280, 330, and 355 mμ. If the transmission at 280 mμ was found to be below 20%, additional readings at 280 mμ were taken on a more dilute sample. Such dilution was permissible because Beer's law was found to hold for all tyrosinase samples. In the case of very crude samples it was found necessary to check their absorption at wavelengths below 280 mμ in order to determine whether or not an absorption maximum due to phenolic substrate material existed in the 260 mμ region of the spectrum. Samples showing an abosrption maximum in the 260 mμ region were found to be unsuitable for a calculation of the protein extinction at 280 mμ by the method described below.

CALCULATIONS

To correct the total extinction (*E*) at 280 mμ for the pigment content, at least 3 readings have to be taken: one at 280 mμ and 2 at selected wavelengths between 330 and 370 mμ. To simplify the final calculations as much as possible the wavelengths 330 mμ and 355 mμ were chosen. As pointed out earlier, the absorption at the two latter wavelengths is due to the pigment content of the solution.

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[5] The activities of the enzyme tyrosinase in catalyzing the aerobic oxidation of the monohydric phenol, *p*-cresol, and the *o*-dihydric phenol, catechol are referred to as cresolase and catecholase activities, respectively.

If one assumes that the pigment absorption curve between 280 and 370 mμ is linear, the portion of the total extinction at 280 mμ which is due to the pigment can be calculated from the equation of the extrapolated line (see Fig. 3). The protein extinction is then obtained by subtracting the extrapolated pigment value from the observed extinction at 280 mμ, *i.e.*:

$$E'_{\text{prot., 280 m}\mu} = E'_{\text{obs., 280 m}\mu} - E'_{\text{pig., 280 m}\mu},$$

where

$$E' = E \times \text{dilution} = (\log \%T_o/\%T) \times \text{dilution}.$$
$$\%T = \%\ \text{transmission as read directly from the instrument.}$$
$$\%T_o = 100\%\ \text{transmission.}$$

As shown in the legend of Fig. 3,

$$E'_{\text{pig., 280 m}\mu} = 3E_{\text{330 m}\mu} - 2E'_{\text{355 m}\mu}$$

and therefore

$$E'_{\text{prot., 280 m}\mu} = E'_{\text{obs., 280 m}\mu} - 3E'_{\text{330 m}\mu} + 2E'_{\text{355 m}\mu}.$$

RESULTS AND CONCLUSIONS

The experimental results, as summarized in Table I and Figs. 1, 2, and 3, have previously been discussed in connection with developing the method. It should be emphasized, however, that enzyme samples showing a phenolic absorption maximum at wavelengths below 280 mμ (see Curve 1, Fig. 1) were not satisfactory for the determination of $E'_{\text{prot.}}$ at 280 mμ by the method of extrapolation.

The enzyme data given in Table II for preparations C259 and C260 are included to show how the catecholase and cresolase activities and the dry weights change with each successive stage throughout the purification process. It can be calculated from the activity data that the purification process employed was that leading to high catecholase preparations.[6]

The spectrophotometric data given in Table II show that except for the first few samples (those having a phenolic absorption maximum at wavelengths below 280 mμ) the calculated value of $E'_{\text{prot.}}$ for each sample taken throughout the purification process was directly proportional to the concentration of the undialyzable solid matter in the sample as determined by the dry weight measurements. The 7 samples taken at successive stages in the purification of enzyme C259 resulted in a protein extinction coefficient having a mean value of 1.37 with an a.d. of ±0.09. The 8 samples of preparation C260 gave a mean value of 1.45 with an a.d. of ±0.09. It can be seen that the difference between the two mean values (0.08 ± 0.13) is not experimentally significant,

[6] Tyrosinase preparations having a high catecholase/cresolase activity ratio are called high catecholase preparations.

and thus the mean of all the determinations (1.41 ± 0.09) may be used as the 280 mμ protein extinction coefficient for judging the purity of mushroom tyrosinase samples throughout the purification procedure. In cases where it may be desired to compare purity levels with those previously reported on the basis of dry weight measurements, the corrected protein extinction at 280 mμ divided by the factor 1.41 will yield the dry weight with an a.d. of ±6.9%. This reproducibility is comparable to that of the direct dry weight determination.

The results also indicate that, because the extinction coefficient of the pigment is much higher than that of the protein at 280 mμ (compare Tables I and II), the relative amount of pigment present is very small at the dilutions used for measuring the extinction. Relatively much more protein is present in terms of dry weight. For this reason, a correction for the absorption of the pigment is essential but a correction for its weight can be neglected.

As pointed out earlier, one may also conclude from these results that the absorption of the pigment material is linear (within an a.d. of ±6.9%) over the range from 280 to 360 mμ, and the extinction coefficients of the enzyme and non-enzyme proteins of mushroom tyrosinase are not significantly different.

The results of several nitrogen determinations on selected dry weight residues (see Table III) indicate that a factor of 9.96 may be used to convert the value of $E'_{\text{prot.}}$, 280 mμ into mg.N/cc. with an a.d. of ±4.3%.

TABLE III

Showing the Constant Relationship between E'_{prot}, 280 mμ and the Nitrogen Content

Enzyme no.	Sample no.	Nitrogen	E'prot./mg.N/cc.
C259	5	0.50	9.76
	9	1.59	8.70
	10	1.07	9.85
	11	0.90	9.79
C260	3	0.54	10.00
	5	0.25	10.35
	9	0.96	10.30
	11	0.73	10.90

Mean: 9.96 a.d. = ±0.43 = ±4.3%

ACKNOWLEDGMENT

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SUMMARY

A spectrophotometric method is described for determining the protein concentration of pigmented mushroom tyrosinase solutions. The method is based on the selective absorption of colorless protein in the 280 mμ region of the spectrum. The pigments present in the aqueous mushroom extracts show nonselective absorption in this region and their contribution to the observed absorption at 280 mμ may be corrected for by extrapolation.

It has been demonstrated that, once the phenolic impurities have been removed in the preliminary purification steps, a constant relationship exists between the corrected protein extinction at 280 mμ, and both the dry weight of undialyzable material and the nitrogen content of enzyme samples taken throughout the purification procedure. The method of enzyme purification is outlined.

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Sweet Potato Phenolase. Preparation, Properties, and Determination of Protein Content

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INTRODUCTION

In a previous communication from these laboratories (1) a spectrophotometric method is described for the determination of the protein content of tyrosinase preparations from the common mushroom (*Psalliota campestris*). To determine whether the same method could be applied to pigmented tyrosinase solutions from other sources, the phenolase present in the sweet potato (*Ipomoea batatas*) has been investigated.

The sweet potato phenolase has attracted the interest of workers in this field because of the fact that fresh slices of the sweet potato show pronounced cresolase activity but only slight catecholase activity[2] (2, 3) when these experimental substrates, *p*-cresol and catechol, are added directly to the intact slices suspended in buffer. However, treatment of the slices with a number of chemical agents, such as acetone, alcohol (2), NaCl (3), destroys the aerobic respiration of the slices partially or completely and markedly increases the catecholase activity. Thus far, the methods used to destroy the cell and prepare the extracts, have yielded enzyme extracts which show catecholase but no cresolase activity (4).

No serious attempt has been made heretofore to purify the phenolase of the sweet potato. It seemed of considerable value, therefore, in connection with the spectrophotometric protein determination studies, to attempt a purification that would enable a comparison of the sweet potato enzyme with the mushroom tyrosinase. The latter is known to be a copper protein; and its mode of action, specificities, pH, and substrate optima, and inhibitors have been carefully studied (5).

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[2] The activities of the enzyme tyrosinase in catalyzing the aerobic oxidation of the monohydric phenol, *p*-cresol, and the *o*-dihydric phenol, catechol, are referred to as cresolase and catecholase activities, respectively.

PURIFICATION OF THE ENZYME

The method of purification described below gives the best results of several procedures that were investigated. It has been found to be reproducible and yields an enzyme about 100 times purer than the acetone-dried powders used by previous investigators (4, 6). Attempts to use the crude acetone powders (6) as a starting point for the purification, resulted in poor yields of an enzyme of lower purity.

Step 1: Crude Juice. Twenty kg. of sweet potatoes[3] were sliced, minced, and the pulp pressed to remove the juice. The 9500 cc. of juice obtained thus had a total catecholase activity of 2,280,000 units and a purity level of 5 units/mg. dry weight.[4] The cresolase activity of the fresh juice was very low, giving a ratio of about 1000:1 for the catecholase to cresolase units/cc. of juice. The juice was dark brown in color and turbid in appearance.

Step 2: Clarification and Concentration of the Enzyme Solution. The juice was clarified and some of the extraneous proteins were removed by adding solid $(NH_4)_2SO_4$ to 0.3 saturation at room temperature (210 g./l. of juice). The precipitate was removed by suction filtration[5] and the clarified filtrate had a volume of 9000 cc., a total activity of 990,000 catecholase units and a dry weight of 6.89 mg./cc. The filtrate possessed no measurable cresolase activity. All of the protein matter in the clarified juice was precipitated by adding solid $(NH_4)_2SO_4$ to 0.7 saturation, and the precipitate was redissolved in water to give a more concentrated dark brown enzyme solution of 2000 cc. volume. This solution contained 840,000 catecholase units and 27.4 mg. protein /cc. (15.3 units/mg.).

Step 3: Fractionation by Ammonium Sulphate. One-half of the above solution (1000 cc.) was fractionated with $(NH_4)_2SO_4$ in the following way: Enough $(NH_4)_2SO_4$ was added to the enzyme solution at room temperature to make it 0.1 saturated with respect to the salt. After stirring for a few min. the resulting protein precipitate was removed by suction filtration and the filtrate was treated with additional $(NH_4)_2SO_4$ to make it 0.2 saturated. Each of the precipitates obtained in this way through successive additions of $(NH_4)_2SO_4$ (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 saturated) were dissolved in water and the resulting enzyme solutions (samples III-A (0.1) to III-A

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[3] The sweet potatoes used in this investigation were the L-5 variety supplied by the U. S. Sugar Experimental Station in Clewiston, Florida.

[4] The catecholase activity was measured using the chronometric method (7) (see footnote to Table V). The cresolase activity was measured by the method of Gregg and Nelson (8). Dry weight measurements were made as described by Mallette (9).

[5] All suction filtrations throughout the course of the preparation, were carried out using a quarter inch pad of Celite (Johns-Manville No. 535) on a filter paper in the Buchner funnel. In all cases, except where stated otherwise, the precipitated protein was dissolved off the Celite pad by stirring in water for 5–10 min. and refiltering, using suction.

(0.6)) were assayed for catecholase activity and for protein concentration, using the spectrophotometric method (1). Dry weight determinations were also made on each sample (see Tables I and II). The filtrate from the final 0.6 saturated solution had negligible activity.

Identical treatment of the other half of the solution from Step 2 gave six more fractions (samples III-B (0.1) to III-B (0.6)) (see Tables I and II).

As can be seen from Table I, 3 of the fractions (A (0.6), B (0.5), and B (0.6)) were considerably purer (activity per unit dry weight) than the others. These 3 fractions were combined and purified as described below, leading to samples IV-A, V-A, and VI-A. The 3 fractions (Table I) of intermediate purity (A (0.4), A (0.5), and B (0.4)) were likewise combined and purified in a similar manner, leading to samples IV-B, V-B, and VI-B Because of their relatively low order of purity, the first 3 fractions obtained in both III-A and III-B were not further purified.

TABLE I

Step III: Fractionation by Ammonium Sulphate

	Saturation	Volume (cc.)	Total catecholase units	Units/mg.
A	0.1	130	31,200	10
	0.2	200	106,000	10
	0.3	150	39,750	15
	0.4	100	55,000	37
	0.5	100	42,000	102
	0.6	100	42,000	200
B	0.1	150	34,500	10
	0.2	175	84,000	10
	0.3	130	54,000	14
	0.4	67	71,200	54
	0.5	45	63,000	280
	0.6	45	63,000	240

Step 4: Dialysis. To remove the contaminating $(NH_4)_2SO_4$, the fractions obtained in both parts of Step 3 which had an activity of 200 units/mg. or better (A (0.6), B (0.5), and B (0.6)) were combined and dialyzed overnight against cold running water. The dialyzed solution (sample IV-A) contained 139,000 catecholase units and 2.64 mg./cc. (229 units/mg.).

Step 5: Lead Subacetate Treatment. A 0.1 saturated solution of lead subacetate (1) was added dropwise to sample IV-A until appreciable precipitate formed. Although the precipitate, when redissolved, gave a solution containing considerable enzyme activity, it was not used further in the purification. Previous experience had shown that it was very difficult to separate the enzyme from the extraneous protein and colored material at this stage. The filtrate from the lead subacetate treatment had a volume of 225 cc., was nearly colorless and contained 78,800 catecholase units and 1.25 mg./cc. dry weight (280 units/mg. dry weight) (sample V-A).

Step 6: Adsorption to Alumina and Fractional Elution. Sample V-A was treated with enough alumina reagent (1) to adsorb all of the activity, and the resulting precipitate was then filtered off and fractionally eluted, using Na_2HPO_4 of increasing concentrations. Each elution was performed by stirring the precipitate for about 15–30 min. with the phosphate solution and then filtering by suction without addition of any further filter aid (samples VI-A-1 to VI-A-5).

The first two eluates obtained, using 10 cc. of 0.04 and 0.09 *M* phosphate, respectively, contained enzyme of relatively low purity, *i.e.*, below 100 units/mg. dry weight. Further elution with 20 cc. of 0.16 molar phosphate gave a third eluate still lower in purity than the sample V-A (starting material). However, the succeeding two eluates (VI-A-4 and VI-A-5) obtained by elution with 20 cc. of 0.2 *M* and with 32 cc. of 0.4 *M* phosphate possessed an activity of 450 and 600 catecholase units/mg. of dry weight, respectively, and each of these contained 16% of the total activity of sample V-A. Both of these fractions were slightly colored and contained 0.05% copper (see Table V.).

The same procedure as described in Steps 4, 5, and 6 was also applied to the samples of intermediate purity resulting from the $(NH_4)_2SO_4$ fractionation in Step 3, giving parallel results and leading to samples IV-B, V-B, and VI-B (see Table II). The best enzyme obtained in the B series was sample VI-B-5, having an activity of 310 units/mg. and containing 0.04% copper (see Table V).

DETERMINATION OF PROTEIN CONCENTRATION

At each stage of the purification procedure described above for the sweet potato oxidase, samples were assayed for dry weight and examined spectrophotometrically in the ultraviolet region of the spectrum.[6] Certain of the samples were also assayed for their nitrogen content.

To determine whether the nature of the protein and pigment absorption in the ultraviolet region was such that a correction for pigment absorption could be applied by extrapolation (1), the complete absorption curves in this region were determined on several samples. The absorption curves for 3 samples at different stages of purification (see Fig. 1) show that a sharp protein absorption band at 280 mμ develops during the purification procedure and the absorption due to the pigment is practically linear in nature over the region of the spectrum between 330 and 355 mμ. For all samples showing a maximum absorption at 280 mμ the amount of the absorption due to protein at this wavelength was obtained by making measurements at 280, 330, and

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[6] All light absorption measurements were made using a Beckman Model DU spectrophotometer with ultraviolet attachment, employing 1 cm. quartz cells and using water or phosphate buffer as a blank.

355 mμ and calculating the value of $E'_{\text{protein, 280 m}\mu}$ by the extrapolative method previously described (1).

In Table II the values of $E'_{\text{protein, 280 m}\mu}$ are compared with the dry weight of the samples. The results show that a reasonably constant relationship exists throughout the purification procedure and a conversion factor of 1.27 ± 0.08 may be used to convert the value of E'_{protein} at 280 mμ into mg. dry weight/cc. It should be mentioned that data similar to those shown in Table II were also obtained on more than 20 samples taken during the purification of two other sweet potato oxidase preparations (trial preparative experiments) and the results led to a conversion factor of 1.25 ± 0.08.

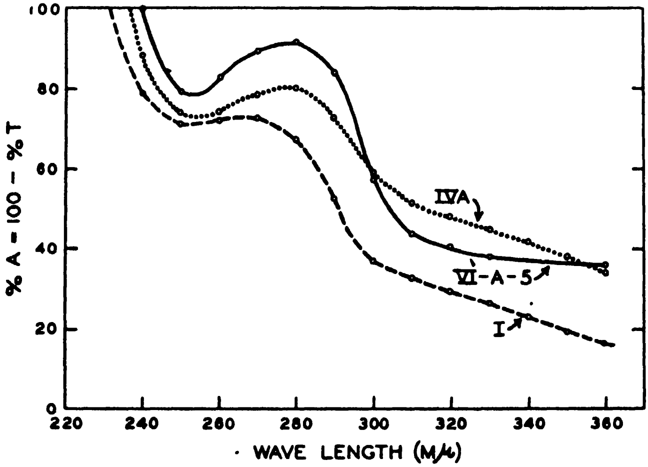

FIG. 1. Showing the development of the protein absorption maximum at 280 mμ during successive stages of purification of the sweet potato phenolase. The curve numbers correspond to enzyme samples in the purification procedure (see text).

The results obtained on those samples which were assayed for nitrogen content are shown in Table III. They reveal that a conversion factor 8.97 ± 0.49 may be used to derive the value of mg. N/cc. from $E'_{\text{protein, 280 m}\mu}$.

The conversion factor which relates the value of $E'_{\text{protein, 280 m}\mu}$ to the dry weight of the sample is slightly lower in the case of the sweet potato phenolase than in the case of mushroom tyrosinase (1.27 as compared to 1.41) (1). This difference in the conversion factor from one source of the enzyme to another makes it necessary to determine

TABLE II

Showing the Constant Relationship between $E'_{\text{protein, } 280\ m\mu}$ *and the Dry Weight*

Sample number	E'protein	Dry weight mg./cc.	E'protein/mg./cc.
III-A (0.4)	19.96	15.00	1.33
III-A (0.5)	5.42	4.12	1.31
III-A (0.6)	2.92	2.34	1.25
III-B (0.4)	22.61	19.60	1.15
III-B (0.5)	6.10	5.00	1.22
III-B (0.6)	7.15	5.82	1.23
IV-A	2.95	2.64	1.12
IV-B	11.69	8.92	1.31
V-A	1.58	1.35	1.17
V-B	2.95	2.40	1.32
VI-A-3	6.85	5.50	1.24
VI-A-4	1.83	1.32	1.39
VI-A-5	0.82	0.67	1.22
VI-B-1	4.94	3.62	1.36
VI-B-2	16.02	11.50	1.39
VI-B-3	8.54	7.43	1.15
VI-B-4	4.18	2.93	1.43
VI-B-5	1.56	1.20	1.30

Mean 1.27 ±0.08 (a.d.)
±6.3%

The method of calculating the values of E'_{protein} is illustrated below, using the data obtained on sample VI-B-1; where $E' = (\log T_o/T)$.

λ	%T	log %T	log T_o/T	Dil.	E'	$3E'$ 330 mμ	$2E'$ 355mμ
280	29.5	1.4698	0.5302	1:10	5.30		
330	52.9	1.7235	0.2765	1:1	0.28	0.84	
355	58.2	1.7649	0.2351	1:1	0.24		0.48

$$E'_{\text{prot.}} = E'_{280\ m\mu} - 3E'_{330\ m\mu} + 2E'_{355\ m\mu} = 4.94.$$

the relationship between the value of $E'_{\text{protein, } 280\ m\mu}$ and the concentration of undialyzable solid material each time that the spectrophotometric method is to be applied to tyrosinase isolated from a new source.

The advantage of the spectrophotometric method lies in its speed and simplicity of manipulations. Once the conversion factor has been established the development of a purification procedure for the enzyme

TABLE III

Showing the Constant Relationship between $E'_{\text{protein, 280 m}\mu}$ and the Nitrogen Content

Sample number	Nitrogen mg./cc.	E'protein/mg.N/cc.
IV-A	0.36	8.20
IV-B	1.29	9.05
V-A	0.18	8.78
V-B	0.35	8.43
VI-A-3	0.72	9.50
VI-A-4	0.20	9.17
VI-B-1	0.50	9.87
VI-B-3	1.07	8.00
VI-B-4	0.44	9.50
VI-B-5	0.17	9.20
		Mean: 8.97 ±0.49 (a.d.) ±5.5%

can be accomplished in a much shorter time than is possible when dry weights are determined by the direct gravimetric method, for the latter requires a long period of dialysis prior to each measurement.

PROPERTIES OF THE SWEET POTATO PHENOLASE

Specificity and Activity

Graubard (4) and Adams and Nelson (10) have reported that the phenol oxidizing enzyme present in aqueous extracts of the sweet potato (*Ipomoea batatas*) possesses the ability to catalyze the aerobic oxidation of *o*-dihydric phenols but cannot bring about the oxidation of monohydric phenols, such as *p*-cresol and tyrosine. As a result, the enzyme has been called catecholase (4) and orthophenolase (11, 12).

Inasmuch as the specificity investigations of Graubard and of Adams and Nelson were preliminary in nature, employing only crude enzyme extracts, it seemed of value to repeat and extend the specificity investigations, employing one of the partially purified sweet potato enzyme preparations described above. For this purpose preparation VI-A-4 was used and the aerobic oxidations which it catalyzed were followed by measuring the oxygen uptake in a respirometer of the Warburg type 25°C. A variety of mono-, di-, and trihydric phenols were investigated. The experimental details are given in the legend of Table IV. In cases where no oxidation was apparent the flasks were shaken for periods up to 2 hr. or more to insure the absence of a long induction period in the reaction (characteristic of monophenol oxidation (13)). In such cases the experiments were always repeated, using much larger amounts of the enzyme and higher substrate concentrations.

The following monophenols were investigated with the purified enzyme: phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2,4-dimethylphenol, and tyrosine. In each case no oxidation was observed over a period of 2 hr., either by oxygen uptake or coloring in the reaction flask. The experiments were run using about 2 manometric units of the enzyme and an amount of substrate equivalent to 2 mg. of *p*-cresol. The addition of a trace of catechol to the *p*-cresol-sweet potato phenolase system, with the intention of removing a possible long induction period (13), resulted only in an oxygen uptake equivalent to the catechol added. Increasing the amount of enzyme used to as high as 100 units in the flask and the amount of substrate to 20 mg. in the flask (where solubility permitted, otherwise 4 cc. of a saturated solution was used) did not alter the results.

TABLE IV

Showing the Relative Reactivities of the o-Dihydric Phenolic Substrates Oxidized by the Sweet Potato Enzyme

Substrate	Enzyme used mg. (dry wt.) $\times 10^2$	Oxygen uptake		QO_2 (manometric) mm.^{3}/mg./hr.
		mm.^{3}/min. during first 5 min.	Total mm.^{3} after 30 min.	
Chlorogenic acid	0.48	24.0	146	300,000
Homocatechol	0.88	21.5	122	146,400
Catechol	2.64	21.8	70	49,500
Protocatechuic acid	13.2	25.3	300	11,500
Pyrogallol	13.2	19.1	127	8,700
Adrenaline	13.2	16.4	90	7,400
Dopa	13.2	13.9	87	6,300

The 8.0 cc. reaction volumes (10.0 cc. in the case of chlorogenic acid) contained in each case 2.0 cc. of (0.1 *M*) citrate (0.2 *M*) phosphate buffer (pH 6.4), 1 cc. aqueous gelatin solution (5 mg.), 1 cc. diluted enzyme solution and an amount of *o*-dihydric phenol equivalent to 20 mg. catechol. All experiments were run at least in duplicate using Warburg respirometers at 25°C. The flasks and contents were brought to temperature equilibrium with the thermostat by shaking slowly for 10 min. prior to closing the manometers, dumping the enzyme from the sidearm of the flask to initiate the reaction, and increasing the rate of shaking to 120 oscillations/min. In the case of chlorogenic acid, protocatechuic acid, adrenaline, and dopa, the center well of the respirometer flasks contained a small piece of filter paper saturated with 0.2 cc. of 20% KOH to absorb any CO_2 that might be liberated (see also footnote to Table V).

In a similar manner the purified enzyme was found to be completely inert to the following polyphenols: resorcinol, hydroquinone, phloroglucinol, 2,3-naphthol, and 1,2-naphthol. Because of their very low solubility, the naphthols were used as saturated solutions (4 cc.). In the case of the 1,2-naphthol slight coloration was observed, but no oxygen uptake could be measured. Likewise, no oxidation could be detected when the purified enzyme was added to systems containing ascorbic acid, *o*-phenylenediamine, 4-nitrocatechol, and 3,5-dinitrocatechol.

The purified enzyme was found to be very active in catalyzing the aerobic oxidation of the following *o*-dihydric phenols: catechol, homocatechol, protocatechuic acid, chlorogenic acid, pyrogallol, adrenaline, and dopa.

Of these 7 compounds, chlorogenic acid was found to be by far the most reactive substrate for the purified enzyme (compare Q_{O_2} values, Table IV). This point is of considerable interest in view of the fact that Rudkin and Nelson (6) have recently isolated chlorogenic acid from the sweet potato and have shown that it has an important function in the respiratory process of the tuber.

During the oxidation of the substrates listed in Table IV, the enzyme was rapidly inactivated, as evidenced by a progressive decrease in the rate of oxygen uptake, most noticeable during the 5–30 min. period of the reaction.[7]

In all cases except that of protocatechuic acid the oxygen uptake had stopped prior to the 30 min. reading (enzyme inactivated). The protocatechuic acid oxidation at the 30 min. period was proceeding at a rate about 20% of its original rate. In the cases of catechol, adrenaline, and dopa, the oxidation had stopped prior to the 15 min. reading.

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[7] Because of the great differences in reactivity of these catechol compounds as substrates for the enzyme, it was not possible to compare them using a constant amount of the enzyme in the reaction flask. For example, an amount of enzyme which gave a suitable rate of oxidation for catechol (within the range of 10–25 mm.^{3}/min., *i.e.*, 1.0–2.5 manometric units) was much too large an amount for the oxidation of chlorogenic acid and too small an amount for adrenaline or dopa. Consequently, the comparisons were made by using such amounts of enzyme that the rate of oxidation during the first 5 min. in each case was within the range of 10 to 25 mm.^{3}/min. Only during this early part of the reaction, and within this range of rate of oxygen uptake, was the reaction course found to be sufficiently linear in nature to allow a satisfactory determination of the rate of oxygen uptake. The rate of oxidation, as determined in this manner for each of the substrates has been converted to Q_{O_2} values for comparison purposes (see Table IV).

The total oxygen absorption in 30 min. (see Column 4, Table IV) corresponds in each case to the oxidation of only a small fraction of the substrate available to the enzyme. It can be seen from the data in the table that there is no simple relationship between the rate of oxidation (during the early stages of the reaction) and the amount of oxidation that a given quantity of the enzyme will bring about. Both the activity of the enzyme and its rate of reaction inactivation vary from one substrate to another; but not in the same way. In this respect the potato enzyme is similar to the purified mushroom tyrosinase (5, 14).

Influence of the Substrate Concentration on the Activity

The data given in Fig. 2 show how the rate of oxidation of catechol, as catalyzed by a constant amount of the sweet potato oxidase (IV-A-4) is influenced by the amount of catechol in the reaction system.

It is apparent that under these conditions the enzyme is most effective in catalyzing the oxidation of catechol when the amount of catechol in the system is within the range of 40–80 mg. (corresponding to a catechol concentration of 5–10 mg./cc. of reaction volume). A similar optimum catechol concentration range was observed when the rate of catechol oxidation was followed by the chronometric method (7).

A dependency of the rate of oxidation on the concentration of the substrate was also observed with the other catechol compounds listed in Table IV. However, the relatively low solubility of chlorogenic acid, protocatechuic acid, adrenaline, and dopa, made it impossible to determine their optimum concentration range. The solubility was exceeded in each case before the maximum rate of oxidation was achieved. It is for this reason that the rates of enzymatic oxidation of these catechol compounds were compared (see Table IV) using an amount of substrate equivalent to 20 mg. of catechol; an amount below the catechol optimum range.

Influence of pH on the Activity

Manometric measurements of the rate of oxidation of catechol as catalyzed by the sweet potato enzyme were made over the pH range 2.2 to 6.8. Higher pH ranges were not investigated because of the oxidation of catechol that occurs in slightly alkaline solutions. The results are shown in Fig. 3.

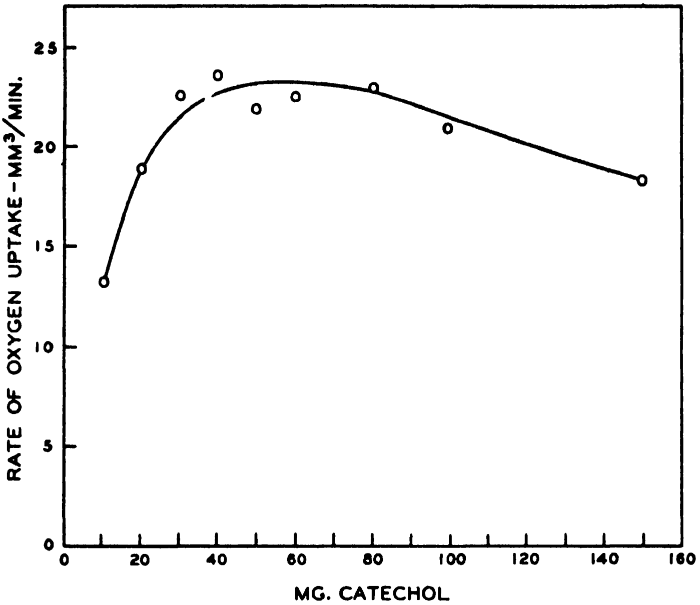

FIG. 2. Showing how the activity of the sweet potato oxidase at pH 6.4 varies with the amount of substrate (catechol) in the reaction system. All rate measurements were made in duplicate at 25°C. using a respirometer of the Warburg type. The reaction flask contained in each case 1.0 cc. of enzyme VI-A-4 (diluted 1:60, *i.e.*, 2.2×10^{-2} mg. dry wt.), 2.0 cc. of (0.1 *M*) citrate (0.2 *M*) phosphate buffer (pH 6.4), 1.0 cc. aqueous solution of gelatin (5 mg.), and 4.0 cc. of catechol solution. The enzyme was added from the sidearm of the respirometer flasks to initiate the reactions after a 10 min. temperature equilibration period. Readings of oxygen uptake were made every minute during the first 5 min. of the reaction; manometers shaking at 120 oscillations /min.

As can be seen from the figure, the rate of oxygen uptake, for a given amount of the enzyme and in the optimum range of catechol concentration, is markedly dependent on the pH of the system. The enzyme is only 50% as effective at pH 2.2 as it is at pH 6.8, and there is no significant variation of its activity over the pH range 6 to 7. All of the activity studies described in this communication were carried out at pH 6.4.

Copper Content

The copper contents of the 3 most active samples of the sweet potato oxidase were determined by the manometric method of Warburg and

Krebs (15), using the procedure described in detail by Lovett-Janison and Nelson (16). To remove extraneous cupric ion, all enzyme samples were dialyzed for 75–90 hr. against Cu-free water prior to the analyses. The dialyses were carried out at refrigerator temperature (5–10°C.) against several changes of Cu-free water. No loss in enzyme activity during the dialysis was observed. The presence of iron in the dialyzed samples could not be detected by the *o*-phenanthroline method (17, 18). The copper and activity data are given in Table V.

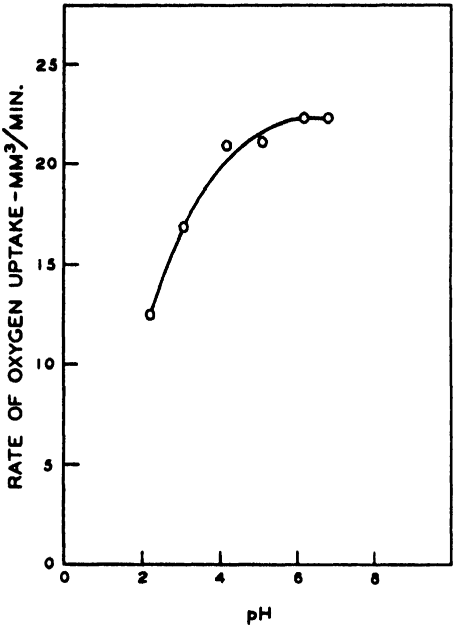

FIG. 3. Showing how the activity of the sweet potato phenolase on catechol varies with pH. All rate measurements were made in duplicate at 25°C. using a respirometer of the Warburg type and employing 2.2 × 10^{-2} mg. of enzyme VI-4-A and 40 mg. of catechol (optimum range) in an 8.0 cc. reaction volume. In each case the reaction system contained 2.0 cc. of (0.1 *M*) citrate (0.2 *M*) phosphate buffer of the desired pH. Other experimental details are given in the legend of Fig. 2.

Additional information concerning the role of copper in the activity of the partially purified sample VI-A-4 was obtained by investigating the inhibitory effect of KCN and of diethyldithiocarbamate on the enzymatic oxidation of catechol. The experiments were carried out manometrically at 25°C. (pH 6.4) using 40 mg. of catechol (optimum range) and 0.05 mg. of the inhibitor in the respirometer flasks. An inhibition in the rate of oxygen uptake of 60% was observed in the case of KCN, and 54% in the case of diethyldithiocarbamate. In each series of experiments the amount of enzyme employed corresponded to 2.4 catecholase units under control conditions.

The data in Table V are insufficient to clearly establish a dependency of activity on the copper content. Further purification of the enzyme (in terms of activity/mg. dry weight) must be accomplished before the relationship between the activity and the copper content can be definitely established. However, these data, in conjunction with the results of the inhibition studies, using two well known cooper inhibitors, make it appear likely that copper plays an essential role in the activity of the sweet potato oxidase, just as it does in the case of other plant phenolases (5, 19).

TABLE V

The Copper Contents and Activities of Three Samples of the Oxidase Prepared from the Sweet Potato, Ipomoea batatas

Enzyme	Copper content			Catecholase activity		
	γ Cu/cc.	Dry wt. mg./cc.	% Cu	Units/cc	Units/mg.	Units/γ Cu
VI-A-5	0.34	0.67	0.051	400	600	1178
VI-A-4	0.69	1.32	0.052	600	450	870
VI-B-5	0.48	1.20	0.040	375	310	782

Catecholase activity determined using the chronometric method (7). The catecholase activity as measured manometrically is always much lower than that obtained by the chronometric method (frequently by a factor of 3–4) when extensive inactivation of the enzyme occurs during the first minute of the enzymatic oxidation of catechol. The chronometric method gives a measurement of the rate of oxidation at zero time (before inactivation) whereas the manometric units are based on the rate of oxygen uptake observed during the first 5 min. of the reaction and are, therefore, markedly dependent on the extent of inactivation occurring during that period.

Comparison of the Sweet Potato Phenolase with Mushroom Tyrosinase

Although both enzymes appear to be copper proteins, and possess the ability to catalyze the aerobic oxidation of certain phenolic bodies (phenolase activity) the sweet potato oxidase differs from mushroom tyrosinase in several important respects. The most striking difference is in specificity. Whereas purified mushroom tyrosinase preparations possess both monophenolase and *o*-dihydric phenolase activity, the partially purified sweet potato enzyme catalyzes only the oxidation of certain *o*-dihydric phenols. The monophenolase activity that can be demonstrated in the intact slices of the sweet potato is lost as soon as the cell is destroyed. It does not appear to be possible to purify the monophenolase activity as in the case of the mushroom enzyme. It is true that the ratio of the two activities of mushroom tyrosinase can be varied in favor of the catecholase activity, but it appears to be due to the alteration of a single homogeneous protein rather than the separation of two different protein enzymes (9).

TABLE VI

Comparing the Activities of Mushroom Tyrosinase and Sweet Potato Phenolase on Certain o-Dihyric Phenols

Substrate	Mushroom tyrosinase[a]			Sweet potato enz.
	Enzyme used mg. × 10^3	Oxygen uptake cm.^{3}/min. during first 5 min.	Q_{O_2} (manometric) cm.^{3}/mg./hr.	Q_{O_2} (manometric) (from Table V)
Catechol	1.40	19.5	83,700	49,500
Homocatechol	2.33	13.9	35,900	146,400
Dopa	3.50	17.3	29,700	6,300
Chlorogenic acid	5.60	21.6	23,200	300,000
Protocatechuic acid	17.5	6.0	2,100	11,500

[a] Tyrosinase preparation C260A14B obtained from the common mushroom (*Psalliota campestris*) by procedures similar to those previously published (1, 20). This enzyme possessed a catecholase activity of 570 units/mg. dry weight (chronometric units, see legend of Table V) and was therefore comparable in purity to the sweet potato enzymes VI-A-4 and VI-A-5 (see Table V). Its catecholase to cresolase activity ratio was 15.4. The manometric experiments were carried out as described in the legend of Table IV.

Of equal interest is the fact that the two enzymes show a markedly different specificity toward *o*-dihydric phenols. This conclusion is supported by the data given in Table VI obtained using the sweet potato enzyme VI-A-4 and a mushroom tyrosinase preparation of comparable purity. A comparison of the Q_{O_2} values for the two enzymes reveals that, whereas mushroom tyrosinase is most effective in catalyzing the oxidation of catechol, and is considerably less effective with chlorogenic acid, the reverse holds true for the sweet potato oxidase. A similar reverse relationship is also evident on comparing catechol and homocatechol or dopa and protocatechuic acid as substrates for the two enzymes.

Although both enzymes show optimum activity within the same pH range when catechol is used as the experimental substrate, the sweet potato phenolase requires a much higher concentration of catechol for optimum activity than does the mushroom enzyme of low cresolase activity.[8] Both enzymes are rapidly inactivated during the oxidation of catechol, and inactivation is also observed during the oxidation of other *o*-dihydric phenols. For both enzymes, the rate of reaction inactivation varies from one substrate to another, but not in the same order.

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SUMMARY

The spectrophotometric method developed for the determination of the protein concentration of pigmented mushroom tyrosinase solutions has been successfully applied to phenolase preparations obtained from the sweet potato (*Ipomoea batatas*). A method leading to the partial purification of the phenolase is described for the first time.

The enzymatic properties of the purified sweet potato phenolase have been investigated and evidence is advanced that the enzyme is a copper-protein.

The more important enzymatic properties have been compared with those of a mushroom tyrosinase of comparable purity.

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[8] Tyrosinase preparations possessing a relatively high cresolase to catecholase activity ratio require a higher concentration of catechol for optimum catecholase activity than do high catecholase tyrosinase preparations (7, 21).

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Biochemical Individuality. II. Variation in the Urinary Excretion of Lysine, Threonine, Leucine, and Arginine[1]

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INTRODUCTION

The development of highly sensitive microbiological procedures for the assay of amino acids has resulted in a number of studies of the urinary excretion of amino acids by humans (1–5). In none of these investigations, however, has attention been focused primarily upon the question of individual differences in amino acid excretion. Our aim was to determine whether individuals show distinctive patterns of amino acid excretion. A somewhat similar approach has been used by Brewer *et al.* (6) in a study of the total amino acid nitrogen content of blood plasma.

We have limited our investigation to 5 subjects and the determination of 4 amino acids, before and after acid hydrolysis of the urine samples. The amino acids studied were lysine, threonine, leucine and arginine.

EXPERIMENTAL

Subjects

Five subjects, all in good health and ranging in age from 22–29, were employed in these studies. The two females are designated F_1 and F_2, the three males, M_1, M_2, and M_3.

Collection and Treatment of Samples

Twenty-four hour urine samples were collected and preserved with toluene. Total volume and specific gravity were measured. Appropriate aliquots were assayed directly for "free" amino acid content. For determination of total amino acid content ("free" plus "bound") a portion of the urine sample was hydrolyzed with 10% HCl by autoclaving overnight at 15 lb. steam pressure. The hydrolyzed sample was evaporated under reduced pressure to remove hydrogen chloride and the residue dissolved in water. Seven urine samples were collected from each of the subjects, usually at intervals of 1–2 weeks.

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[1] This is one of a series of studies to be carried on in this laboratory, the implications of which are briefly outlined in the first publication, (1) Williams, R. J., *Chem. Eng. News* **25**, 1112 (1947), and in a more extended way in (2) Williams, R. J., *Quart. J. Studies Alc.* **7**, 567 (1947), and (3) Williams, R. J., The Human Frontier, Harcourt, Brace and Co., New York, 1946.

Dietary Control

Two of the urine samples from each subject were collected while the subject was on an army K-ration diet.[2] The diet was maintained for 4 consecutive days and samples collected on each of the last 2 days. Water intake was not controlled. No dietary control was exercised during the collection of the remaining 5 samples from each subject, except that unusual dietary excesses were avoided.

Assay Procedures

The 4 amino acids were determined by microbiological assay, utilizing the organism *Strep. faecalis*, and the procedure of Stokes *et al.* (7). Growth was measured turbidimetrically after 40 hr. incubation. Considerable difficulty was encountered in the assay of certain of the urine samples which contained small quantities of amino acids and relatively large quantities of substances toxic to the test organism. This difficulty was circumvented by the use of a modified assay procedure which has been described in a previous paper (8). All amino acid values reported are based on assays at several levels of urine concentration such that the growth response obtained represented a substantial fraction of the maximum possible growth. Assay values calculated from different sample levels agreed within ±10%, and usually within much narrower limits.

DISCUSSION

Tables I and II show the complete data for lysine. The data were placed on a body weight basis in view of an obvious correlation, in the case of most of the amino acids, between the amount excreted and the body weight of the subject.

The values for bound amino acids were obtained by taking the difference between the assay of the hydrolyzed sample of urine and the untreated sample. That the test organism does not respond appreciably to amino acids in peptide linkage was shown by assaying unhydrolyzed urine samples after treatment with chloramine-T as described by Van Slyke *et al.* (9, 10). This treatment destroys only free amino acids. Samples treated in this manner, when assayed, showed either no growth stimulation or such slight stimulation as to be of no analytical signifi-

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[2] Army K-rations were furnished by the War Department, Quartermaster Food and Container Institute for the Armed Forces.

TABLE I

"Free" Lysine (γ*/day/kg.*)

Sample no.	Subjects				
	F_1	F_2	M_1	M_2	M_3
1	1150	610	320	260	290
2	2030	530	350	380	420
3	880	590	280	290	220
4	1810	700	350	270	340
5	1410	420	290	270	200
6	1720	530	340	300	380
7	1820	470	330	450	430

TABLE II

"Bound" Lysine (γ*/day/kg.*)

Sample no.	Subjects				
	F_1	F_2	M_1	M_2	M_3
1	710	890	670	750	610
2	1220	1060	990	930	960
3	1210	1250	1040	940	830
4	620	1120	950	640	690
5	630	910	820	730	430
6	1070	910	830	860	490
7	870	870	760	1050	1230

cance. Certainly in excess of 90% of the growth stimulation obtained in the assay of untreated urine is due to substances inactivated by chloramine-T.

Samples 6 and 7 in Tables I and II were collected while the subjects were on a K-ration diet. Because these data and all other data obtained while subjects were on a K-ration diet do not seem to differ from the general excretion pattern without dietary control, we have felt justified, even without a statistical test of significance, in combining all of the data obtained for a given subject, regardless of diet.

The statistically summarized data for all 4 amino acids are shown in Tables III and IV. It is clear from a gross inspection of these data that the subjects exhibit a certain individuality. Thus, the urine samples of

TABLE III

Excretion of "Free" Amino Acids

Results expressed in γ/day/kg. body wt.

7 samples per subject

Subject	Lysine		Threonine		Leucine		Arginine	
	Mean excretion	Standard deviation	Mean excretion	Standard deviation	Mean excretion	Standard deviation	Mean excretion	Standard deviation
F_1	1546	413	327	69	111	21	106	26
F_2	550	93	276	60	97	24	103	25
M_1	323	28	403	84	89	21	90	19
M_2	317	71	403	51	108	9	91	20
M_3	326	92	194	75	76	35	76	18
χ^2 values[a]		42.0 (7.37 excluding F_1)		1.62		8.98		1.23
F values	–		11.7		2.58		2.07	

[a] For description of statistical methods employed see Snedecor, Statistical Methods, 4th Ed., The Iowa State College Press, 1946.

TABLE IV

Excretion of "Bound" Amino Acids

Results expressed in γ/day/kg. body wt.

7 samples per subject

Subject	Lysine		Threonine		Leucine		Arginine	
	Mean excretion	Standard deviation	Mean excretion	Standard deviation	Mean excretion	Standard deviation	Mean excretion	Standard deviation
F_1	904	263	389	69	289	36	370	56
F_2	1001	144	384	62	307	56	420	36
M_1	866	133	351	114	274	44	297	30
M_2	843	143	410	29	276	46	326	49
M_3	749	281	383	116	293	113	346	149
χ^2 values		6.11		11.1		10.5		20.5 (2.45 excluding M_3)
F values	1.43		0.43		0.30		—	

subject F_1 are characterized by a high mean for free lysine excretion and a high variability in the amount excreted. Subject F_2, compared to the male subjects, also shows a high mean for free lysine excretion and a high mean for bound arginine excretion. Subject M_3 exhibits a low mean for free threonine excretion and a marked variability in the excertion of bound arginine. Subjects M_1 and M_2 have very similar excretion patterns characterized by a high mean for free threonine excretion. They differ from the other 3 subjects in the particulars previously noted.

These gross observations are born out by statistical treatment of the data as indicated by the χ^2 values and variance ratios noted in Tables III and IV. Each of the columns of standard deviations was tested for homogeneity by Bartlett's method. The χ^2 values with 4 degrees of freedom indicate a lack of homogeneity in the case of free lysine and bound arginine. This inhomogeneity, as indicated by the χ^2 values in Tables III and IV, disappeared upon elimination of the F_1 data for free lysine and the M_3 data for bound arginine.

Variance ratios, F-values, with 4 and 24 degrees of freedom, were calculated for the columns of means in Tables III and IV, except for the cases of free lysine and bound arginine, which require special treatment because of their heterogeneous variance. The value of 11.7 for free threonine is significant at the 1% level. The value of 2.58 for free leucine just falls short of significance at the 5% level, which is 2.69.

In the case of free lysine, excluding the F_1 data, a variance ratio of 15.84 with 3 and 24 degrees of freedom is obtained, indicating that the subjects differ significantly in their means at the 1% level. The difference here is obviously attributable to F_2. The difference between the means of F_1 and F_2 was tested by the *t* test (11) with results indicating a significant difference at the 1% level.

In the case of bound arginine, exclusion of the M_3 data permits the calculation of a variance ratio of 10.41 with 3 and 24 degrees of freedom. Consideration of the 3 individual degrees of freedom reveals that the difference between the means of M_2 and M_3 is not significant, the difference between the means of F_1 and F_2 is significant at the 5% level, and the difference between the means of the men and women is significant at the 1% level. The mean of M_3, who is excluded from the analysis of variance, was compared with the pooled mean of the other two men by means of the *t* test, and the difference found to be not significant. It should be noted that, in the case of both free lysine and arginine, the women have significantly higher means than the

The data obtained in this investigation do not permit of any valid inferences as to the cause of the individuality which is observed. The correlation between body weight and amino acid excretion has already been mentioned. This correlation was very clearly shown by scatter diagrams, plotting amino acid excretion (mg./day) *vs.* body weight. When this is done, a positive correlation is obtained in every case except that of free lysine excretion.

The amino acid excretion data reported herewith show satisfactory agreement with those reported by Dunn *et al.* (4) and by Woodson *et al.* (5). The data of Steele *et al.* (1), obtained from subjects on highly restrictive diets, are in most cases much lower. The individual variation in amino acid excretion noted in this study is supported by the data of Woodson *et al.* (5), showing the results of analyses on a single sample from each of 18 subjects.

Acknowledgment

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Summary

Seven 24 hr. urine samples from each of 5 subjects were assayed for 4 amino acids (lysine, threonine, leucine, and arginine) in the free and bound state. The excretion of these amino acids by a given subject was not markedly altered when a normal uncontrolled diet was replaced by an army K-ration diet.

Gross inspection of the data suggests the existence of individual patterns of amino acid excretion. Statistically significant differences in the mean were found in the excretion of free lysine, free threonine, and bound arginine. Except for the excretion of free lysine, there is a positive correlation between amino acid excretion and body weight.

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Fermentation of Tyrosine by Marine Bacteria [1]

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INTRODUCTION

The bacterial degradation of tyrosine has been shown by several workers to give rise to phenolic compounds. Traetta Mosca (7) reported the formation of *p*-cresol with a culture resembling *Pseudomonas aeruginosa,* while Rhein (6) and Janke (3) obtained *p*-cresol with an organism described as *Clostridium cresologenes.* Phenol has resulted from the fermentation of tyrosine with coliform bacteria by Berthelot (1), Rhein (6) and others. Various acidic products have also been isolated, such as *p*-hydroxyphenylpropoinic acid, *p*-hydroxyphenylacetic acid, and *p*-hydroxybenzoic acid (6,2). Traetta Mosca reported benzene from fermentation by a *Pseudomonas* culture from mud, but the identification was based solely on organoleptic tests.

Since many geologists (10) believe that marine sediments, containing plant and animal proteins, may be the source beds of petroleum, the decomposition products of tyrosine are of particular interest. The benzene ring of this amino acid has been suggested as one of the sources of aromatic nuclei in petroleum. A study of tyrosine breakdown was undertaken to determine the chief paths of attack by marine bacteria and whether the end products paralleled those reported by earlier workers from common fresh-water bacteria.

EXPERIMENTAL

Mixed cultures of marine origin capable of utilizing tyrosine as a source of carbon were developed by inoculating marine mud samples[3] into an enriched sea water medium. The samples used were obtained from recent marine sediments collected off the coast of southern California in the vicinity of Sorrento Slough and Sweetwater Estuary. The medium consisted of 4 g. L-tyrosine, 1 g. $Na_2SO_3 \cdot 7H_2O$, 1 g. K_2HPO_4, 40 ml. yeast extract, and 960 ml. sea water. The 40 ml. of yeast extract represented the water extract of 4.5 g. of yeast cells prepared by heating 1 lb. of bakers' yeast with 4 l. of water for 3 hr. on a steam bath, followed by centrifuging and sterilizing the extract. The main source of C and N in the medium was tyrosine. The reaction flasks were placed under an atmosphere of nitrogen and allowed to incubate at 28°C. At 14 day intervals the cultures were transferred to fresh tyrosine-sea water broth.

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[2] This work is part of the activities of Research Project 43B—The Conversion of Organic Materials into Petroleum—sponsored by the American Petroleum Institute.

[3] Marine muds were supplied through the courtesy of Dr. C. E. ZoBell, Scripps Institution of Oceanography, La Jolla, Calif.

Products from Tyrosine Breakdown

One liter batches of tyrosine medium were allowed to ferment for 2 weeks. The broth was brought to pH 8 and extracted with ethyl ether for 8 hr. to remove the ether-soluble neutral compounds. Then the water residue from the extraction was brought to approximately pH 1.0 with H_2SO_4 and extracted with ether a second time. The ether was allowed to evaporate at room temperature to approach constant weight. In experiments in which there was evidence of a volatile acid such as acetic acid or a volatile neutral compound, the ether was removed by a distilling column. The results of the analyses of 3 mixed cultures are shown in Table I. The neutral extracts of 2 of the cultures were found to contain *p*-cresol. The 2 acid extracts that gave an appreciable weight of product proved to be *p*-hydroxyphenylacetic acid.

TABLE I

Products Formed from Tyrosine by Fermentation with Mixed Cultures of Marine Bacteria

(Extract from 1 l.)

Culture	Neutral extract		Acid extract	
	Weight in g.	Compound	Weight in g.	Compound
6J	0.01	—	1.45	*p*-Hydroxyphenylacetic acid
7J	0.61	*p*-Cresol	0.15	Not identified
8J	0.74	*p*-Cresol	1.46	*p*-Hydroxyphenylacetic acid

Description of Bacterial Cultures

The mixed cultures of 7J and 8J were purified by plating on sea water-nutrient agar and transferring representative colonies to tyrosine-sea water broth. Three cultures of those isolated were fairly active on tyrosine. They were gram negative rods, growing well on most salt water media, but showing scant growth on standard laboratory media. The enrichment cultures were facultative anaerobes, growing well either with or without atmospheric oxygen. Under anaerobic conditions the organisms were able

TABLE II

Characterization of Gram Negative Rods from Marine Muds

(A = acid; growth in salt media only)

No. of culture	Gelatin liquef.	Nitrate reduction	Glucose broth	Maltose broth	Sucrose broth	Lactose broth	Mannose broth	H_2S	Motility
7J2	−	+	A	A	A	A	A	−	+
8J3	−	−	A	A	A	±	−	−	+
8J4	−	−	A	A	A	A	−	−	+
603[a]	−	−	A	A	A	−	−	−	+

[a] *Pseudomonas stereotropis* (ZoBell).

to utilize sulfate as a hydrogen acceptor for the oxidation of various substrates and concurrent production of H_2S. With the exception of the salt requirement, the bacteria were in most respects similar to the common gram negative facultative organisms found on plant material and in garden soil. Some of their characteristics are given in Table II.

It will be noted that the characters given here correspond rather closely to one of the organisms isolated by ZoBell (9) from marine mud and named *Pseudomonas stereotropis.*

Identification of Products from Pure Cultures

Fermentation mixtures of 3 pure cultures were set up as before using 0.4% tyrosine in the medium. Since it was observed that the bacteria tend to be surface-active,

TABLE III

Products Formed from Tyrosine by Fermentation with Pure Cultures of Marine Bacteria

(Extract from 1 l.)

Culture	Type medium	Wt. of extract		Neutral products recovered	
		Neutral g.	Acid g.	Compound	Theor. per cent[a]
7J2	Sea water broth	0.32	0.10	*p*-Cresol	14
8J3	Sea water broth	0.35	0.14	Phenol	17
8J4	Sea water broth	0.37	0.20	Phenol	18
7J2	Broth + rocks	0.51	0.09	*p*-Cresol	22
8J3	Broth + rocks	1.09	0.18	Phenol	52
8J4	Broth + rocks	1.21	0.16	Phenol	60

[a] Calculated on basis of 4 g. tyrosine added/l.

TABLE IV

Melting Points of Derivatives of Neutral Extracts, °C.

Compound from	Aryloxyacetic acid	Mixed with known compound	Tribromo-compound	Mixed with known compound
7J2	136	136	—	—
8J3	99	99	93	93
8J4	99	99	93	93
Phenol	99	—	93	—
p-Cresol	136	—	—	—

fermentations were prepared in the usual manner with flasks of broth and also in 10-liter bottles containing crushed limestone rock. The results are given in Table III. The amount of acid extract was small and evidently a mixture of several compounds, so no identification was made from these fractions. In the neutral fractions, *p*-cresol was identified from one culture and phenol from the other two. These compounds occurred in the ether extract in yields equivalent to 60% of the tyrosine added in the presence of crushed rock. The identification of phenol and *p*-cresol by melting points of aryloxyacetic acid and tribromo derivatives is given in Table IV. It is significant that derivatives made from the crude ether extracts had sharp melting points and within the limits of the melting point data proved to be either pure phenol or pure *p*-cresol.

Mechanism of Formation

It has been suggested by Kurona (5) and others that tyrosine was broken down to phenolic compounds by a mechanism similar to that shown below except that *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetaldehyde were also included.

$$\underset{\text{tyrosine}}{HOC_6H_4CH_2CH(NH_2)COOH} \xrightarrow[-NH_3]{O} \underset{p\text{-hydroxyphenylpyruvic acid}}{HOC_6H_4CH_2COCOOH} \xrightarrow[-CO_2]{O}$$

$$\underset{p\text{-hydroxyphenylacetic acid}}{HOC_6H_4CH_2COOH} \xrightarrow{-CO_2} \underset{p\text{-cresol}}{HOC_6H_4CH_3} \xrightarrow{O}$$

$$\underset{p\text{-hydroxybenzaldehye}}{HOC_6H_4CHO} \xrightarrow{O} \underset{p\text{-hydroxybenzoic acid}}{HOC_6H_4COOH} \xrightarrow{-CO_2} \underset{\text{phenol}}{C_6H_5OH}$$

The conversion of tyrosine to *p*-hydroxyphenylpyruvic acid is in accord with present knowledge on amino acid breakdown. Both *p*-hydroxyphenylacetic acid and *p*-cresol have been isolated from tyrosine fermentation (2,6).

A series of experiments was set up to attempt to ferment certain possible intermediates in the breakdown of tyrosine to *p*-cresol and phenol. The results are shown in Table V. When tyramine was used, only traces of an unidentified phenolic compound were found. This evidence is against the possibility that an initial decarboxylation to tyramine may be a step in the formation of *p*-cresol. In the case of *p*-hydroxyphenylacetic acid, *p*-cresol was formed in significant amounts by Culture 7J2, the same organism that produced it from tyrosine. The phenol-producing cultures apparently were not able to form any phenol from *p*-hydroxybenzoic acid. The last compound bears the same relation to phenol as *p*-hydroxyphenylacetic acid does to *p*-cresol.

It would appear from these results that *p*-hydroxyphenylacetic acid functions as an intermediary in the formation of *p*-cresol. But the formation of phenol, which is a more common end product of tyrosine breakdown, does not go by way of this acid. This observation is in agreement with Rhein (6), who found that a phenol-producing strain of *Escherichia coli* would not form phenol from *p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpyruvic acid, or tyramine. However, Rhein did report a test for phenol from *p*-hydroxybenzoic acid, which we were

TABLE V

Neutral Compounds Produced from L-*Tyrosine and Possible Intermediates in the Formation of Phenol and p-Cresol*

Substrate	7J2	Organism 8J3	8J4
L-Tyrosine	*p*-Cresol	Phenol	Phenol
Tyramine	Unknown compound (trace)	Unknown compound (trace)	Unknown compound (trace)
p-Hydroxphenylacetic acid	*p*-Cresol	Unknown compound (trace)	Unknown compound (trace)
p-Cresol	None	None	None
p-Hydroxybenzaldehyde	None	None	None
p-Hydroxybenzoic acid	None	None	None

unable to duplicate with our organisms. Nor were we able to detect any phenol from *p*-hydroxybenzaldehyde or *p*-cresol.

The lack of activity of the two cultures on these compounds begins to exhaust the chemical possibilities of a simple decarboxylation and stepwise oxidation of tyrosine to phenol. In a study of the bacterial degradation of tryptophan to indole, Krebs and coworkers (4) could find no compound containing the intact indole nucleus which would act as a precursor of indole, and proposed that the nitrogen-containing ring is broken and later closed to form indole. The lack of precursors for indole production is noteworthy, because the same side chain, α-alanine is involved. There is no corresponding evidence, however, to suggest that the benzene ring is broken and reclosed to form phenol. There is the possibility that the organisms may be able to split off the entire side chain as a compound of 3 carbon atoms, leaving only hydrogen on the ring in addition to the *p*-hydroxy group. Analagous evidence for this type of split has been found for tryptophan by Wood and coworkers (8) using tryptophanase prepared from *E. coli*. They state that the products of tryptophan degradation are equimolar quantities of indole, pyruvate, and ammonia.

The production of phenol and indole, or *p*-cresol and skatole, illustrates the removal of an oxygen-containing side chain leaving intact the original aromatic ring or ring plus a methyl group. Although in neither case has a hydrocarbon resulted, such reactions are of considerable interest as they may provide clues to a mechanism for the biological formation of hydrocarbons.

SUMMARY

1. Three bacterial cultures were isolated from marine sediments; two cultures formed phenol and one formed *p*-cresol from *L*-tyrosine.

2. Fermentation studies indicate that *p*-hydroxyphenylacetic acid may function as an intermediate in the formation of *p*-cresol, but not in the formation of phenol. No compound other than tyrosine was found to give rise to significant amounts of phenol.

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A Simplified Technique for a Large Scale Crystallization of Human Oxyhemoglobin. Isomorphous Transformations of Hemoglobin and Myoglobin in the Crystalline State [1]

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INTRODUCTION

In the original crystallization of human hemoglobin (1), the bulk of the pigment precipitated in an ill-defined crystalline form in the cellulose sac upon short dialysis at refrigerator temperature against either saturated $(NH_4)_2SO_4$ or 2.8 M phosphate buffer. The concentrated solution above the precipitate was used for the crystallization, while the precipitate itself was discarded (1). This process was very wasteful of material, and it could be questioned whether the pigment in the precipitate and that crystallized from the concentrated supernatant fluid were identical hemoglobins, although it was shown that solutions of the two were indistinguishable spectrophotometrically (1).

It has now been found that all the pigment present in the hemolyzed solutions prepared from the separated corpuscles of citrated human blood (1) can be obtained in beautifully crystalline and stable form in the dialysis sac. By means of the simplified new procedure 100 g. or more of unaltered crystalline human oxyhemoglobin can be prepared easily and quickly. The stability of the preparations has permitted a study of crystalline isomorphism.

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[1] This work was done under contract between the Office of Naval Research and the University of Pennsylvania. Brief mention of the work was made in a report at the Sir Joseph Barcroft Memorial Conference on Hemoglobin, Cambridge, England, June 15–17, 1948.

METHODS

New Crystallization Procedure

A stroma-free solution (8–10 mM/l. in hemoglobin concentration, referred to a molecular weight of 16,700) was prepared from cells recently separated by centrifugation from fresh citrated adult human blood. The blood was collected for a blood bank, and contained approximately 75 ml. of a 3.2% solution of sodium citrate dihydrate (slightly more than isotonic) per 400 ml. of blood (see *Addendum*). The earlier communication (1) may be consulted for details. The improvement in technique consisted in providing conditions which *delay precipitation during dialysis* in the refrigerator of the stroma-free solution against 2.8 M phosphate buffer, pH 6.8 (371 g. of $K_2HPO_4 \cdot 3H_2O$ and 160 g. of KH_2PO_4, made up to 1 liter).

The dialysis was carried out in sacs of Visking cellulose sausage casing. A convenient size of sac was 13 in. long, made from casing of 1⅝ in. diam. (1). The dialysis was in 2 steps. First, the hemoglobin solution was dialyzed in the refrigerator at a temperature of 4°C. against *warmed buffer*. The buffer solution was previously warmed to 37°C., and a ratio of 1:3 volumes within and outside of the sac was used. Under these conditions, in contrast with dialysis against unwarmed phosphate buffer or saturated $(NH_4)_2SO_4$ (1), no precipitation occurred in the sac during 6–10 hr. of dialysis in the refrigerator.[2] At this stage the temperature of the solution and dialyzate was 4°C. The dialyzate was now replaced with an equal volume of fresh unwarmed 2.8 M phosphate buffer. Upon continued dialysis in the refrigerator for about 12–24 hr., crystallization of practically all of the pigment was accomplished within the sac.

Metmyoglobin was obtained in crystalline form from horse cardiac muscle by a modification in the combined methods of Theorell (2) and Morgan (3). The essential steps in the procedure were the isolation of the pigment in 3 M phosphate buffer, pH 6.6 (350 g. of $K_2HPO_4 \cdot 3H_2O$ and 200 g. of KH_2PO_4, made up to 1 l.), followed by the dialysis of this solution at 4°C. against saturated $(NH_4)_2SO_4$ (700 g. of the salt plus 1 l. of water, final volume of about 1390 ml.). To permit large growth on the walls of the cellulose sacs, dialysis was continued for several weeks to several months in the refrigerator. Details will be presented separately.

Crystallography and Photomicrography

Crystallographic characterization was by means of the petrographic microscope. Numerous photomicrographs in color were taken by the writer on Kodachrome Professional Type B film, 3¼ × 4¼ in., from selected typical examples of which the accompanying colored plates were made. For photomicrographic recording, either the petrographic or ordinary microscopes were used in conjunction with a Zeiss-Hegener camera. The latter has extension bellows permitting the use of low power objectives, which are desirable for good definition and detail in human hemoglobin crystals owing to their depth and intensity of pigmentation. For this reason also, smaller crystals in fresh suspensions on the microscope slide were chosen for photography. The illuminating source was a 500-watt G. E. Mazda projection lamp, with voltage controlled for correct color temperature by means of a variable slide wire resistance and voltmeter. The correct exposure time was derived by the application of suitable correction factors (determined photometrically) to readings obtained at the highest levels of illumination on a Weston Master II exposure meter, held at 1 in. above the microscope ocular.

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[2] The temperature of the solution in the sac does not exceed 29°C., and this level may be reached during only a brief interval of time. Any effect of elevated temperature upon hemoglobin alteration is hence precluded.

Spectrophotometry

The exact character of the pigments originally crystallized and those produced by transformation in the crystalline state was determined spectrophotometrically. The measurements were made both on the crystalline suspensions as well as on solutions prepared from them by the addition of water. Dilute solutions were examined in the usual 1 cm. cuvette, while concentrated solutions were measured in the Drabkin and Austin 0.007 cm. cuvette (4).

RESULTS

Crystallographic Character of Human Hemoglobin

Examination, following suspension of the crystals in added 2.8 *M* phosphate buffer of pH 6.8, revealed that the crystallization was complete and uniform, with the suspending medium free of color. The pH of solutions, prepared from the crystals by dilution with water, was 6.8, determined with the glass electrode. The earlier preparations, crystallized in the presence of $(NH_4)_2SO_4$, had a pH of about 5.9 (1).

Most of the crystals varied in size from a fraction of a mm. to individual macro-sized crystals as large as 3 mm. along their long, *c* axis (1). The crystalline preparations were unusually stable in the refrigerator. Cyanmethemoglobin (or ferrihemoglobin cyanide) in the crystalline form (see below) was particularly stable. A crystalline suspension in a vial, carried about for more than 6 months in the writer's vest pocket, has shown neither alteration in physical form nor in spectroscopic properties.

The *crystal habit* was that of well-developed, symmetrical bipyramids, with twinning very frequent early in the crystallization (Upper Fig., Plate 1). The axial ratios were practically identical with those previously reported, as were also the *negative* birefringence in all orientations except the isotropic "square" base (1).

State of the Crystallized Human Oxyhemoglobin

From the chemical as well as physiological viewpoints the most important facts about the crystallized hemoglobin are that it was unaltered from its state in the original oxygenated blood, and that it was *oxyhemoglobin*, free of all except at present indeterminable small quantities of such derived pigments as methemoglobin or ferrihemoglobin (5). These conclusions were reached from the practical identity of the spectrophotometric constants (ϵ values[3]) obtained on hemolyzed whole human blood and on solutions prepared from the original crystallized material. These absorption values need not be given here, since they agree, within the 0.5% error of the method of measurement, with those already reported (1) for our earlier crystallizations.

TABLE I

Agreement in Total Pigment Concentration ("Total Hemoglobin"), Measured Spectrophotometrically as Oxyhemoglogin, and, After Conversion, as Deoxygenated Hemoglobin, Methemoglobin and Cyanmethemoglobin

In all cases the values are in m*M*/l. of the solution adjusted to pH 7.2 and measured in the 0.007 cm. cuvette (4). Concentration was calculated from *D* (*optical density* corrected to a depth of 1 cm.) for the particular sample divided by the appropriate mean ϵ value, used as an absorption constant (see footnote 3). The standard ϵ values employed were obtained from whole, hemolyzed blood (1). They were 15.34, 8.50, and 14.61 for HbO_2 at 578, 562, and 542 mμ, respectively, 13.55 for Hb at 555 mμ, 4.38, 4.35, and 6.70 for MHb (pH 7.2) at 578, 562, and 542 mμ, respectively, and 11.50 for MHbCN at 540 mμ.

Experiment no.	Material	Total pigment measured as			
		HbO_2[a]	Hb[b]	MHb[c]	MHbCN[d]
1	Stroma-free solution	9.26	9.23	9.30	9.23
2	Crystallized material (fresh)	11.19	11.22	11.24	11.20
3	Crystallized material (fresh)	20.44	20.37	20.50	20.49
4	Crystallized material (4 weeks old)	10.35[e]	10.86	10.92	10.90

[a] Original material.

[b] Deoxygenated by addition of solid $Na_2S_2O_4$.

[c] Solid ferricyanide added.

[d] Solid ferricyanide and cyanide added.

[e] 6.7% of MHb, present in original sample, accounts for low value when measured as HbO_2.

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[3] Our ϵ values may be defined as *fractional molecular extinction coefficients* (6), and are for a concentration of 1 m*M*/l. (referred to an equivalent weight of 16,700 in the case of hemoglobin) and a depth of 1 cm. Thus, $\epsilon = [1/(c \times d)] \times \log I_0/I$, where the concentration c is expressed in m*M*/l., the depth d in cm., the original intensity I_0 is 1.0, and the intensity of transmitted light I is expressed as a fraction of unity. The spectral interval (band width) employed was 1.5–2.0 mμ.

We previously reported (5) that methemoglobin could not be demonstrated spectrophotometrically in many samples which we examined, consisting of arterial blood from normal dogs as well as normal human subjects. It was concluded, therefore, that if the oxidized pigment is present in normal arterial blood its concentration must be less than 0.5% of the "total hemoglobin." Later, a similar conclusion was reached by Van Slyke *et al.* (7) on the basis of gasometric measurements. The excellent agreement in the values, recorded in Table I, for the total pigment concentration, measured as HbO_2 (a solution of the original, oxygenated crystallized material), Hb (after deoxygenation by means of solid $Na_2S_2O_4$ added to the crystalline suspension), MHb at pH 7.2 (after addition of solid ferricyanide), and MHbCN (after conversion in the crystalline state with solid ferricyanide and cyanide), indicates unequivocally that, in the crystallized preparations also, measureable amounts of ferri- or methemoglobin were not revealed. Spectrophotometrically the crystalline material was unaltered, "pure" oxyhemoglobin.

Crystalline Character of Horse Metmyoglobin

Reports on previous crystallizations of myoglobin (2, 3) indicate that the crystals were not fully developed, and they have been described as "sheaves of needles" (2). This is the typical habit of the crystals which first form. However, in the present crystallization, growth has resulted in exceptionally well-defined clusters (1 mm. to larger than 3 mm. in size) of lath-like, highly pleochromatic plates or prisms (Upper Fig., Plate 3). The individual crystals were strongly birefringent and had straight extinction.

Isomorphous Transformations in the Crystalline State

We had found (1) that the blood pigment of man, crystallized as HbO_2 in the presence of $(NH_4)_2SO_4$ at a pH of approximately 5.9, was converted slowly (and spontaneously) into MHb, without any apparent change in the crystal system, so that methemoglobin was a crystallographic pseudomorph of oxyhemoglobin. Such a spontaneous conversion of HbO_2 to MHb also occurred in the crystalline state with the present preparations in 2.8 *M* phosphate buffer, pH 6.8, but the oxidation appeared to be appreciably slower than in the former crystallization. On standing in the refrigerator, measurable amounts of MHb were found only after about 2 weeks, and increased thereafter to 70–80% conversion of HbO_2 to MHb in 6 months. The slower formation of MHb in the new crystallizations is probably mainly accounted for by the more alkaline pH of the suspension (8).

The most striking characteristic of the present crystals was that they could be suspended, at room temperature, in any desired amount of fresh 2.8 *M* phosphate buffer of pH 6.8 without alteration of crystalline character. This stable behavior permitted a study of the effect of various solid reagents (ferricyanide, $Na_2S_2O_4$, and ferricyanide plus cyanide) added to the crystalline suspension. In this way the isomorphous transformation of human oxyhemoglobin in the crystalline state (Upper Fig., Plate 1) has been demonstrated to ferri- or methemoglobin (Lower Fig., Plate 1), to deoxygenated hemoglobin, and to cyanmethemoglobin (Upper Fig., Plate 2). These crystallographic isomorphs possessed the color and spectra characteristic of and associated with their individual solutions. They were completely stable, except for the deoxygenated or reduced hemoglobin which exhibited a tendency, during an observation period of an hour or longer, to degenerate from its original crystalline form.[4]

Examples of isomorphous transformation in the crystalline state are by no means confined to the oxyhemoglobin of man. Granick (9) has demonstrated such a transformation in the case of guinea pig HbO_2 to MHb, and later we reported (1) the conversion of horse monoclinic HbO_2 to MHb. In the Lower Fig., Plate 2, a large monoclinic crystal of horse cyanmethemoglobin is shown. Originally the blood pigment of the horse had been crystallized in the presence of oxalate (1) as monoclinic HbO_2. On standing, the crystals were converted isomorphically into monoclinic MHb, and thence, by means of the addition of solid cyanide, into monoclinic MHbCN. The isomorphous transformation of horse metmyoglobin into cyanmetmyoglobin is illustrated in the Upper and Lower Fig., Plate 3.

Perutz and Weisz (10) have reported that crystals of human hemoglobin, similar in type to ours (1), were secured by Mrs. H. M. Jope by dialysis against 3.5 *M* $(NH_4)_2SO_4$ (462 g./l. of the salt), which were stable at room temperature in 3.8 *M* $(NH_4)_2SO_4$ (500 g./l., a saturated solution of the salt). We have been unable to verify this particular observation. Even the very cautious addition of 3.8 *M* $(NH_4)_2SO_4$ as a diluent to our crystalline material, prepared in the presence of $(NH_4)_2SO_4$ (1), has invariably resulted in departure from crystalline towards amorphous appearance.

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[4] This relative instability of deoxygenated hemoglobin transformed in the crystalline state by the action of hydrosulfite on crystalline oxyhemoglobin may be related to the finding reported by Drs. J. R. P. O'Brien and H. M. Jope (Oxford) and M. Perutz (Cambridge) at the recent Barcroft Memorial Conference on Hemoglobin at Cambridge that the blood pigment of adult man, crystallized as reduced hemoglobin, is *monoclinic*, whereas oxyhemoglobin and methemoglobin are *orthorhombic*.

Discussion

In a decision as to crystal class, greater reliance must be placed upon X-ray crystallographic measurements than on those yielded by the petrographic microscope technique, employed by the writer. From their X-ray data Perutz and Weisz (10) have concluded that the crystals of human carbonyl-, oxy- and methemoglobin, prepared for them by Mrs. H. M. Jope, are not *tetragonal* (as deduced by us for oxyhemoglobin and for methemoglobin, spontaneously transformed in the crystalline state from the former (1)), but *orthorhombic pseudotetragonal* in character. The possibility that our "tetragonal" crystals and the orthorhombic pseudotetragonal of Perutz and Weisz are true polymorphous modifications can be excluded only by subjecting unaltered oxyhemoglobin crystals, obtained exactly by our technique, to X-ray analysis. A decisive conclusion cannot be reached from measurements with the petrographic microscope on crystals suspended in random orientation, since it rests on a decision as to whether the isotropic base of the bipyramids is square or "nearly square." For the present one should accept the view of the Cambridge investigators that the oxyhemoglobin of man belongs to the orthorhombic system of symmetry. However, it is worthy of mention that a more detailed description[5] than given by Perutz and Weisz (10) of the crystallizations of Mrs. H. M. Jope has revealed other than bipyramidal forms. For example, methemoglobin was tetrahedral, with presumably half of the octahedral faces undeveloped, while hexahedral prisms or plates, besides bipyramidal, were present in their oxyhemoglobin specimens.[5] In our fresh crystallizations of oxyhemoglobin (whose unaltered character was established spectrophotometrically) and met- and cyanmethemoglobin, produced isomorphically from the former, the habit was invariably and uniformly bipyramidal.

Summary

A new method is described whereby the hemoglobin of man is easily and quickly crystallized in bulk quantities of 100 g. or more by dialysis against 2.8 M phosphate buffer of pH 6.8.

In contrast with the earlier technique (1), practically all of the pigment present in the stroma-free solution obtained from fresh citrated adult human blood was recovered in crystalline form.

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[5] Report by J. R. P. O'Brien and H. M. Jope at the Cambridge Hemoglobin Conference (see footnote 4).

The crystals were of good size and stability, and were in the habit of beautifully developed symmetrical bipyramids. The nature of the pigment in the original crystalline material was that of unaltered oxyhemoglobin. The presence of methemoglobin, beyond a concentration of less than 0.5 %,was excluded by spectrophotometric analysis.

The isomorphous transformations in the crystalline state of human oxyhemoglobin, horse oxyhemoglobin, and horse metmyoglobin are reported.

ADDENDUM

Since submission of this paper, the writer has found that blood, treated with the ACD anti-coagulant, now frequently employed in collections for blood banks, is not as suitable for the crystallization of hemoglobin as the citrated blood, described in the text. The ACD formula is 14 g. of citric acid monohydrate, 40 g. of sodium citrate dihydrate, and 90 g. of glucose (dextrose)/2900 ml. of aqueous solution. 125 ml. of this solution are used/500 ml. of blood. The exact reasons for the difficulty of crystallizing hemoglobin from blood, treated with ACD, are at present not clear. However, hemoglobin can be crystallized from such blood by a modification in the procedure which has been described. After 24 hr. of dialysis in the refrigerator, (during which time, with ACD blood, little precipitation of hemoglobin occurs), further 2.8 *M* phosphate buffer is added slowly to the dialyzed solution until crystallization. The crystals are small, but typical bipyramids.

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PLATE 1

Upper Figure: Oxyhemoglobin of man. Magnification × 145

Lower Figure: Methemoglobin of man, isomorphically transformed from the crystalline oxyhemoglobin by addition of solid ferricyanide. Magnification × 140

PLATE 2

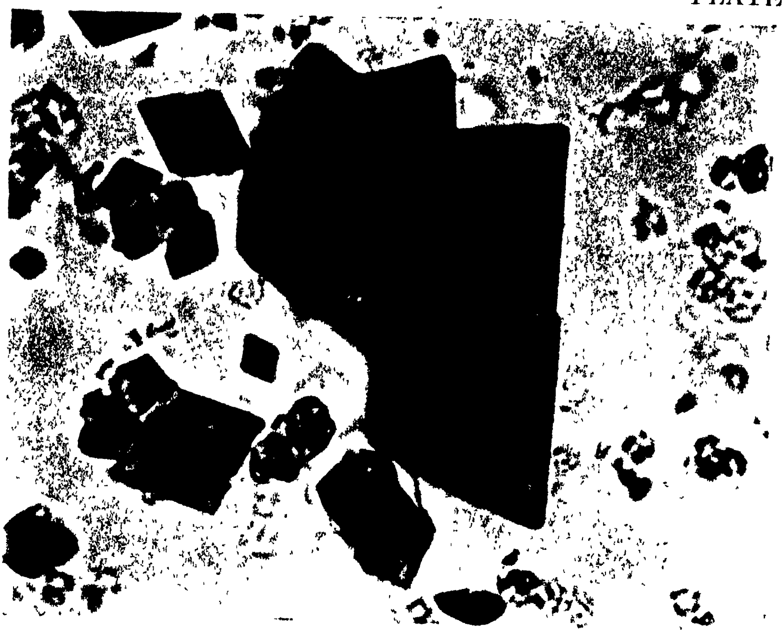
Upper Figure: Cyanmethemoglobin of man, isomorphically transformed from crystalline oxyhemoglobin by addition of solid ferricyanide and cyanide. Magnification × 140

Lower Figure: Cyanmethemoglobin of horse, isomorphically produced in crystalline state from original oxyhemoglobin (see text). Magnification × 110

PLATE 3

Upper Figure: Metmyoglobin of horse. Magnification × 130

Lower Figure: Cyanmetmyoglobin of horse, isomorphically transformed from crystalline netmyoglobin by addition of solid cyanide. Magnification × 150



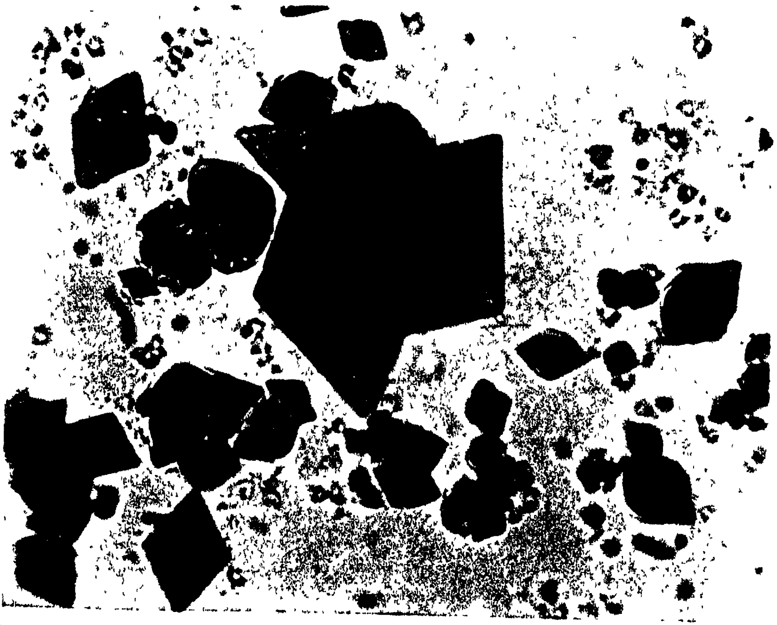


PLATE II

As described before (1), crystalline alkaline phosphatase from kidneys of juvenile rabbits was prepared according to van Thoai *et al.* (4), and the enzymic activity determined by the hydrolysis of sodium phenyl phosphate as described by Buch and Buch (5).

The effect of increasing amounts of α-*dl*-tocopheryl phosphate on the enzyme inhibitory action of the following compounds has been determined: estrone-3-phosphate,[2] estradiol-3,17-diphosphate,[2] progesterone,[2] and phlorizin. As tocopheryl phosphate is precipitated by magnesium ions, the experiments were carried out in the absence of magnesium. The results are presented in Figs. 1 and 2.

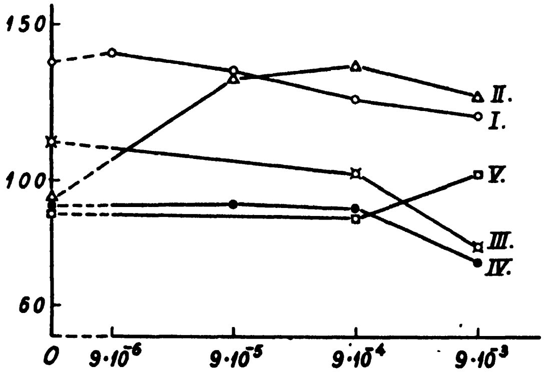


FIG. 2. The effect of tocopheryl phosphate concentration on the inhibition of phosphatase activity. Ordinate: γ phenol liberated after 20 min. incubation. Abscissa: mol. tocopheryl phosphate. Curve I.: Without inhibitor. Curve II.: 1.6×10^{-4} *M* estradiol-3,17-diphosphate. Curve III.: 10^{-2} *M* phlorizin. Curve IV.: 1.6×10^{-4} *M* estrone-3-phosphate. Curve V.: 10^{-3} *M* progesterone. Each point represents the mean of at least 5 determinations.

It appears that tocopheryl phosphate *per se*, in not too small concentrations, slightly inhibits the phosphatase, while together with estradiol-3,17-diphosphate it reverses completely the inhibition by the latter compound. Slight reactivation with greater amounts of tocopheryl phosphate was also observed when the enzyme was inhibited by progesterone. No reactivation has been found in the case of phosphatase inhibition by estrone-3-phosphate and by phlorizin. A discussion of these findings, together with further experiments, *i.e.*, with estradiol-3-monophosphate, will be published later.

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[2] Kindly supplied by A. B. Leo, Hälsingborg, Sweden.

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Incorporation of C^{14} of Glycine into Protein and Lipide Fractions of Homogenates

We recently reported that the incubation of C^{14}-labeled glycine with rat liver homogenate resulted in an uptake of radioactive carbon into the protein fraction (1). Surprisingly, most of the C^{14} bound by the protein was present as serine (2). Subsequent work shows that part of the incorporated C^{14} was associated with lipide, probably phosphatidyl serine. The acetone previously used in our washing procedure did not remove this lipoidal-C^{14} completely from the protein. A complete separation is achieved when trichloroacetic acid (TCA)-washed protein fractions are extracted with hot alcohol-ether, instead of acetone.

Experiments were conducted to permit critical examination of both lipide-free protein and lipide (alcohol-ether) extracts. After 1 hr. incubation with $H_2NC^{14}H_2COOH$, the specific radioactivity of the lipide was twice that of protein. However, there were 20 mg. protein and less than 10 mg. lipide in each experiment, so that about equal amounts of C^{14} went into each component (400 ct./min. or 3% uptake). With $H_2NCH_2C^{14}OOH$, the C^{14} concentrations in lipide and protein were approximately equal. Hence, the former incorporated only half as much total C^{14} as the latter.

The greater uptake of C^{14} by lipide in $H_2NC^{14}H_2COOH$ experiments is due, at least in part, to the fact that COOH-labeled glycine gives rise only to COOH-labeled serine, whereas methylene-labeled glycine yields serine labeled on both α and β carbons (3).

In examining the lipoidal solutions, aliquots were hydrolyzed with 6 *N* HCl in the presence of serine carrier. The latter, after 5 recrystallizations from hot water, accounted for essentially all of the C^{14}. Serine added to unhydrolyzed aliquots removed only 5% of the C^{14}. Glycine carrier removed no C^{14} after acid hydrolysis of the lipides. Treatment of the unhydrolyzed lipides (from $H_2NCH_2C^{14}OOH$ experiments) with

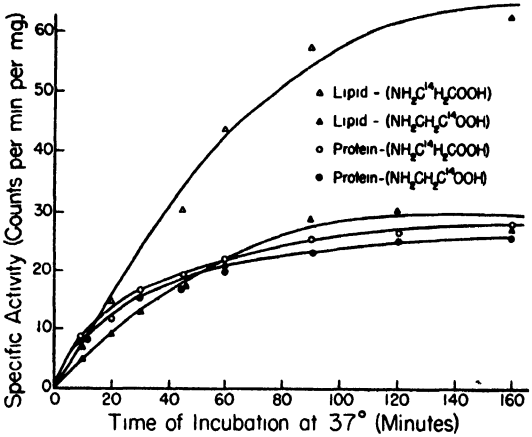

FIG. 1. Incorporation of C^{14} of glycine into protein and lipide of homogenates.

Rat liver was homogenized with one part of 0.9% KCl–0.4% $KHCO_3$ (saturated with 95% O_2–5%CO_2) of pH 7.6. 0.3 ml. aliquots were incubated with 0.03 mg. glycine (12,000 ct./min.) in tubes flushed with O_2–CO_2 and rotated horizontally. Then 6 ml. of 10% TCA were added, the precipitates centrifuged, and washed 3 times with 5% TCA. The protein precipitates were extracted once with alcohol, 3 times with hot 3:1 alcohol-ether, once with ether, and collected on plates for weighing and counting. The combined alcohol and alcohol-ether extracts from each incubation were evaporated to dryness, the residues reëxtracted with alcohol-ether, and the extracts evaporated on plates.

ninhydrin liberated all of the C^{14} as $C^{14}O_2$. These results suggest that the alcohol-ether soluble C^{14} was present in serine bound through its hydroxyl group to lipides. Folch (4) showed that phosphatidyl serine reacts with ninhydrin.

The following observations support the view that the C^{14} uptake by protein represents incorporation of amino acids into protein molecules:

1. No $C^{14}O_2$ was evolved when unhydrolyzed protein (from $H_2NCH_2C^{14}OOH$ experiments) was heated with ninhydrin. All of the C^{14} was released from acid-hydrolyzed protein by ninhydrin.

2. Hydrolysis of protein from $H_2NCH_2C^{14}OOH$ experiments to peptides with crystalline pepsin or trypsin, followed by ninhydrin treatment, released no $C^{14}O_2$. Hydrolysis with trypsin, chymotrypsin, carboxypeptidase, and erepsin, followed by ninhydrin treatment, released 75% of the C^{14}.

3. Carrier experiments with acid-hydrolyzed proteins from either $H_2NC^{14}H_2COOH$ or $H_2NCH_2C^{14}OOH$ incubations showed that about 60% of the C^{14} was due to glycine and 40% to serine.

4. Little C^{14} was lost when the proteins were heated from 15 to 60 min. in 5% TCA. Fifteen min. heating is reported to remove nucleic acids completely (5)

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Xanthurenic Acid Excretion in the Human Subject on a Pyridoxine-Deficient Diet[1]

The demonstration of xanthurenic acid excretion in the pyridoxine-deficient rat (1), mouse (2), dog (3), pig (4), and monkey (5), coupled with the fact that it appears in the urine of the monkey very early during the depletion period led us to a search for the compound in human subjects maintained on a vitamin B_6-deficient regimen for a relatively short time.

Two subjects persisted on a deficient diet for 3 weeks, followed by an additional week during which they received daily supplements of pyridoxine hydrochloride (10 or 15 mg.). A third subject was able to tolerate the deficient diet for a period of one week only.

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[1] Aided by a grant from the California Fruit Growers' Exchange.

The basal diet was a casein-sucrose-corn oil diet which included salt mixture. One of the subjects ate some quantity of milk chocolate and gelatin in addition to the ingredients of the basal diet. Microbiological assay of the chocolate and gelatin for vitamin B_6 showed them to contribute a negligible quantity of the vitamin. The diet was supplemented with vitamins A and D and the known available synthetic vitamins with the exception of *p*-aminobenzoic acid, vitamin K, and α-tocopherol. When the subjects ate sufficient quantities of the diet, they maintained their weights during the course of the experiment and no subjective symptoms were manifested except that one subject expressed that she became fatigued easily during the third week of the deficiency period. Xanthurenic acid excretion was measured by the method of Miller and Baumann (2) before and after 10 g. test doses of DL-tryptophan.[2] These tests were performed during the deficiency periods as well as during periods of supplementation with pyridoxine.

TABLE I

Xanthurenic Acid Excretion

Results expressed in mg./24 hr.

Subject	Dietary status	Pyridoxine supplement	Before tryptophan	After tryptophan
D.B.	Synthetic diet for 21 days	None for 21 days	9.2	271.0
	Syntheitc diet for 28 days	10 mg./day for 7 days	6.2	28.4
H. M.	Natural diet (control period)	5 mg. every other day for 14 days	10.1	33.2
	Synthetic diet for 7 days	None for 7 days	4.3	46.8
	Synthetic diet for 14 days	None for 14 days	7.0	429.0
	Synthetic diet for 21 days	None for 21 days	7.4	514.7
	Synthetic diet for 28 days	15 mg./day for 7 days	5.0	33.1
	Natural diet for 38 days	15 mg./day for 45 days	9.9	38.9
N. K.	Natural diet (control period)	None	11.6	47.0
	Synthetic diet for 6 days	None for 6 days	6.0	31.2

The results are summarized in the table below and show that, although there were small increases in apparent xanthurenic excretion following tryptophan feeding during periods when the subjects were receiving pyridoxine, there was a marked increase in apparent xanthurenic acid after 2 and 3 weeks on the deficient diet. The administration of pyridoxine for one week, following the 3 week deficient period resulted in a return to decidedly lower levels of excretion of xanthurenic acid. Qualitative tests of the urine of the pre- and post-deficient and deficient periods when no tryptophan was fed were negative for xanthurenic acid. This was also true of urines collected during tryptophan feeding while the subjects were receiving pyridoxine.

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[2] Generously supplied by Dow Chemical Company, Midland, Michigan.

The results show that the human subject reacts similarly to a number of other animal species (1–5) to pyridoxine deficiency and develops a derangement of tryptophan metabolism (1) which is manifested by the excretion of xanthurenic acid. This metabolic fault can be detected early in both the monkey (5) and human when extra tryptophan is fed. Our observations point to the fact that the excretion of xanthurenic acid following test doses of tryptophan is one of the earliest detectable manifestations of pyridoxine deficiency.

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Growth of Rats on Hydrolyzed Zein [1]

It was found in earlier experiments (1,2,3), that zein, even when enriched by the addition of amino acids approximately to the level of casein, does not promote satisfactory growth in infantile rats. The reason for this anomaly has been investigated in growth experiments with rats with the following results. The composition of the diets used and the condensed growth results are presented in Table I.

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[1] The expenses of this study were in part defrayed by a grant from the National Vitamin Foundation.

It was found that the addition of 4% zein to a diet containing 12% casein does not interfere with normal growth. Therefore, the presence of a toxic factor in zein or an amino acid imbalance as a reason of failing growth promotion appears unlikely.

We predigested zein *in vitro* with proteolytic pancreatic enzymes (Pancreatin, U. S. P., Armour and Co.) for two days until 65% of the total nitrogen had been liberated in the form of amino nitrogen. Feeding of this predigested zein did not improve the growth; therefore, delayed release of some amino acids during digestion (as suggested in the case of soy bean protein by Melnick, Oser and Weiss, (4)) cannot be responsible for the failure to promote growth.

TABLE I

Growth of Rats During 19 Days and Composition of Diets

Diet fed	18% Zein +82% basal diet	12% Casein +88% basal diet	12% Casein +6% zein +82% basal diet	18% Enzymatically digested zein +82% basal diet	18% Acid hydrolyzed zein +82% basal diet
Average daily growth of rats in grams	0.8	3.8	3.7	0.6	3.2
Average daily food consumption in grams	6.6	8.7	8.5	6.8	9.1

The zein (Zein "A" of Corn Products Refining Co.) and the digested samples were mixed to a *basal* diet which contained also the supplementary amino acids. Its composition was as follows: Corn starch, 3050 g. (78.3%); rice bran concentrate, 400 g. (10.26%); cottonseed oil, 200 g. (5.13%); U.S.P. salt mixture, 200 g. (5.13%); fish oil (1 g. contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 g. (1.27%); riboflavin, 75 mg.; Ca pantothenate, 150 mg.; choline chloride, 2.5 g.; L-arginine 1%; L-histidine 0.5%; L-tryptophan 0.25%; L-lysine 1.2%; L-cystine 0.25%.

In the next experiments, zein was digested for 8 hr. with 6 *N* HCl. After careful removal of the HCl, the zein hydrolyzate was concentrated at low temperature *in vacuo* and mixed with the basal diet. The rats on this diet showed excellent growth.

These experiments indicate that acid hydrolysis liberated some growth factors, probably amino acids, which are not released by either intestinal digestion or *in vitro* enzymatic hydrolysis. The question of whether these resistant linkages are already present in the corn, or are artefacts produced during manufacture of zein is now under investigation. A study of the nature and biological significance of these resistant linkages is also in progress.

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Colorimetric Determination of Folic Acid and Adenine

A method recently described for the colorimetric determination of folic acid (1) involves reduction with metallic zinc in acid solution, thereby liberating an aryl amine which is diazotized and coupled with the Bratton-Marshall reagent (2). In applying the procedure to tissue homogenates, high values were obtained with heart and muscle. It was found that adenine, adenosine triphosphate, and nucleic acid preparations interfered with this determination by producing diazotizable substances on reduction with zinc. No color was observed under these conditions with guanine, cytosine, isocytosine, uracil, thymine, or thiamine. The reduction product of adenine formed an orange-colored derivative with the Bratton-Marshall reagent, having a maximum light absorption at 505 mμ. A linear relationship was observed between optical density and concentration of adenine when measured at this wavelength.

It was found that folic acid could be determined in the presence of adenine without interference by the use of titanous chloride as a reducing agent in place of zinc. The reduction proceeds rapidly at room temperature, and excess titanous reagent is removed by precipitation as the hydrated oxide in alkaline solution. The reduced compound is then acidified, diazotized and coupled, and readings are made at 555 mμ against a reagent blank. The optical density was found to be proportional to folic acid concentration over a wide range, and tissue blanks were much lower than in the zinc procedure. Complete details of the method will be published at an early date.

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The Inactivation of L-Amino Acid Oxidase by Inorganic Phosphate and Arsenate [1]

To our knowledge there is no report in the literature of the inhibition of enzymes by low concentrations of inorganic phosphate ions, although higher concentrations of phosphate have been found inhibitory to certain enzymatic reactions involving phosphate compounds (1–3). We have found that low concentrations of phosphate markedly inhibit the activity of the L-amino acid oxidase of certain snake venoms. The action of phosphate on this enzyme does not appear to follow the laws of substrate competition, nor does it seem to affect the prosthetic group. Our evidence to date indicates that a combination with the enzyme protein is involved.

The inactivation increases with the time of incubation with phosphate, the temperature of incubation, pH, and the molarity of phosphate, and ranges from 0 to 100%, depending on the experimental conditions. It is independent of both the nature and concentration of the substrate used in subsequent assay. There is a rapid initial reaction between the enzyme and phosphate, followed by a slower one, until an apparent equilibrium is reached, which is characteristic of the experimental conditions.

When the molarity of the phosphate is plotted against the per cent inhibition at equilibrium, a typical Michaelis dissociation curve is obtained. Thus, 2.5×10^{-4} *M* phosphate effects 50% inhibition at 30°C., pH 7.2, while 5×10^{-5} *M* phosphate still gives an appreciable effect. The inactivation increases markedly as the pH is raised from about pH 6 to pH 8. For example, with 2×10^{-3} *M* phosphate buffer (temp. = 30°C.) at pH 5.7, only 5% inhibition is obtained at apparent equilibrium, compared to 95% at pH 7.9.

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[1] Supported by the Prentiss Foundation. The help of Miss Barbara Biro is gratefully acknowledged.

Inorganic arsenate quantitatively duplicates the effect of phosphate in every respect. Organic phosphate compounds (Na β-glycerophosphate, phenylphosphate), inorganic pyrophosphate, and arsenite affect the enzyme only slightly or not at all in 10^{-2} *M* concentration

TABLE I

0.7 mg. of venom (dry weight) incubated in Warburg vessels with phosphate buffer at pH 7.2 for 10 min. at 38°C. Controls were incubated similarly but without phosphate. Then L-leucine and tris-(hydroxymethyl)-aminomethane buffer (4) at pH 7.2 were tipped in from the side arm to give final concentrations of 7×10^{-3} *M* and 1×10^{-2} *M*, respectively. O_2 uptake was measured for 30 min. at 38°C. in a final volume of 3 ml. The final molarity of phosphate in the experiments taken from the literature was 6.7×10^{-2}; in our experiments the phosphate concentration during incubation was 4×10^{-2} *M*, except in the case of *A. mokasen*, where it was 2×10^{-1} *M* and *N. naia* where it was 1×10^{-2} *M*.

Source of enzyme	Zoological family	Q_{O_2}			Inhibition
		Literature (5)	In phosphate	No phosphate	
					per cent
Crotalus viridis	*Crotalidae*	270	323	777	58
Crotalus horridus	*Crotalidae*	70	72	227	68
Crotalus adamanteus	*Crotalidae*	50	44	743	94
Crotalus atrox	*Crotalidae*	—	32	416	92
Crotalus terrificus	*Crotalidae*	10	85	296	71
Sistrurus catenatus	*Crotalidae*	100	257	524	51
Agkistrodon piscivorus	*Crotalidae*	380	49	630	92
Agkistrodon mokasen	*Crotalidae*	45	39	234	83
Naia naia	*Elapidae*	100	144	132	0
Naia flava	*Elapidae*	60	69	56	0
Micrurus fulvius	*Elapidae*	—	141	158	11[a]
Bungarus coeruleus	*Elapidae*	250	243	193	0
Echis cantenatus	*Viperidae*	130	196	236	17
Vipera russelli	*Viperidae*	760	602	600	0

[a] Probably represents experimental error as only minute amounts of this venom were available and thus 0.2 mg. enzyme per vessel was used.

over short periods of time (10 min.). However, marked inactivation results when the enzyme is incubated with these substances for 4 hr. at 38°C. or 20 hr. at 30°C. A large number of other anions failed to affect the enzyme in 10 min. at 38°C., but over several hours incubation with the enzyme, in the absence of substrate, certain ions brought about marked inactivation at 10^{-3} *M* or lower concentration (*e.g.*, sulfate, molybdate, ferricyanide, *etc.*). Another group of anions (chloride, bromide, nitrate, azide, and certain organic bases) failed to inactivate the enzymes even at 10^{-2} *M* concentration and over prolonged periods of contact. As a matter of fact, chloride and other non-inhibitory ions can completely prevent the effect of phosphate in equimolar amounts. These ions, however, do not cause reversal and can, therefore, be used to fix the inhibition at any time.

Analogs of the prosthetic group (riboflavin, isoriboflavin) and amino acids in catalytic amounts protect the enzyme from the action of phosphate and arsenate. Reversal has not been accomplished even by exhaustive dialysis.

Although the work summarized above was carried out with moccasin venom (*Agkistrodon piscivorus*) other snake venoms are also inhibited. In the determination of the Q_{O_2}'s (see table), a short reaction time and large excess of phosphate were used to approximate the conditions under which the Q_{O_2}'s available in the literature were obtained (5). Representative venoms of the 3 main families of poisonous snakes have been studied for the sensitivity of their L-amino acid oxidases to phosphate. The enzyme from all *Crotalidae* venoms tested was strongly inhibited by phosphate; the enzyme from *Elapidae* venoms was unaffected or slightly "activated"; and in one member of the *Viperidae* family it was slightly inhibited by phosphate. Other L-amino acid oxidases, from animal kidney (6) and *Proteus vulgaris* (7), were not inhibited by phosphate.

Our studies are being continued in an attempt to determine the mechanism of the reaction.

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Inhibition of Chymotrypsin by Structural Analogs of Specific Substrates

The enzymatic activity of chymotrypsin toward specific peptide or ester substrates (1,2) may be inhibited by analogs which are either devoid of the susceptible peptide (amide) or ester bond, or which contain in its place a bond which itself is resistant to enzymatic hydrolysis. Since the degree of competitive inhibition by a given compound depends, under otherwise identical conditions, on the ratio (3) of the enzyme-substrate dissociation constant, K_m, to the enzyme-inhibitor dissociation constant, K_I, inhibition increasing with increasing ratios, substrates of relatively high (GTA), medium (ATA)[1] and low (BTA) K_m values were employed in testing the inhibitory activities of the present compounds. The relative insolubility of one of these substrates (BTA) in aqueous solutions (2) required some of these measurements to be carried out in the presence of 30% methanol. Representative results are given in the Table.

While the inhibitory activity of benzoylphenylalanine, one of the enzymatic reaction products of the hydrolysis of the phenylalanine analog of BTA, is barely measurable when tested in 30% methanol toward BTA, appreciable inhibition occurs when measured in aqueous solutions toward substrates of lower affinity toward chymotrypsin (ATA, GTA). Calculations show that the low inhibition by compounds 1 and 5 toward BTA is caused by an additional effect of methanol[1] on the ionization of these inhibitors. Benzoyl-D-phenylalanine is more inhibitory than the corresponding L-isomer. The ketone derivative of specific substrates (inhibitor 6), previously reported to be inactive, is

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[1] Hydrolysis of ATA by chymotrypsin is characterized by the following kinetic constants. In aquous solutions, $K_m = 0.023$, $k' = 0.0027$; in 15% methanol, $K_m = 0.036$, $k' = 0.0025$; in 21% methanol, $K_m = 0.068$, $k' = 0.0029$.

TABLE I

Inhibitory Activity of Compounds[a]

Inhibitor (I)	Inhibition toward substrates (S)					
	BTA		ATA		GTA	
	S/I[b]	Inhib.[c]	S/I[b]	Inhib.[c]	S/I[b]	Inhib.[c]
		per cent		*per cent*		*per cent*
1. Benzoyl-DL-phenylalanine	1	1	1	69.1	1	71.4
2. Benzoyl-L-phenylalanine					2	37.6
3. Benzoyl-D-phenylalanine					2	54.1
4. α-Acetaminocinnamic acid	1	13.0	1	36.7		
5. Hydrocinnamic acid	1	3	1	75.2		
6. 1-Phenyl-2-acetamino-butanone-3	1	18	1	56.3		

[a] Measurements were carried out at 25°C. in 0.1 *M* phosphate buffer, pH 7.8. Measurements with BTA as substrate were carried out in 0.045 *M* phosphate buffer, pH 7.8, containing 30% methanol. BTA = benzoyl-L-tyrosineamide, ATA = acetyl-L-tyrosineamide, GTA = glycyl-L-tyrosineamide.

[b] Substrate/inhibitor mole ratio.

[c] Expressed as percentage change of the proteolytic coefficient at constant substrate concentration, caused by the addition of the inhibitor.

The kinetic constants are as follows: K_m in aqueous solutions: GTA = 0.122, ATA = 0.023. K_m in 30% methanol: BTA = 0.0424, ATA (by extrapolation) = 0.167. K_I of benzoyl-DL-phenylalanine in aqueous solutions, measured toward GTA = 0.027.

of considerable inhibitory activity when tested in suitable concentrations toward a substrate of relatively low affinity (ATA). It is of interest to note that hydrocinnamic acid fulfills the structural requirements of specific inhibitors for two proteolytic enzymes, *i.e.*, carboxypeptidase (4) and chymotrypsin. The full account of this work will include a variety of other structural analogs of specific substrates for chymotrypsin.

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Nicotinic Acid in Processed Cigar Tobacco

It has been known for some 80 years that nicotine can be partially oxidized to nicotinic acid by strong oxidizing agents such as conc. HNO_3, H_2SO_4, permanganate or dichromate, preferably in the presence of catalysts, and at temperatures exceeding 100°C. (1).

There are no reports, to the authors' knowledge, on a formation of nicotinic acid from nicotine under much milder conditions, if we exclude the oxidation, by oxygen, of photochemically activated nicotine, which yields, among other products, nicotinic acid (2).

Freshly harvested leaves of cigar tobacco contain considerable amounts of nicotine (1–4% of dry weight), but only small amounts of nicotinic acid ($\overset{=}{<}$ 0.01% of dry weight). During the proper processing of the leaves, particularly during the final operation called "resweat" or "fermentation" (3), their nicotine content drops considerably, by as much as 40–90% of the initial value. An extensive study of the nitrogenous compounds, including the pyridine derivatives in the leaf tissues, and of their fate during the fermentation, has led us to the conclusion that a large part of the disappearing nicotine is transformed into various products of partial oxidation, one of them being nicotinic acid. From the fermented samples, nicotinic acid can be *recovered* in amounts corresponding to 0.2–0.4% of the dry weight.[1] Various other oxidation products of nicotine found in the fermented leaves total about 1.2–1.6% of the dry weight. Generally, between 50 and 70% of the disappearing nicotine can be accounted for by the sum of these oxygen-containing pyridine derivatives. The amounts of nicotinic acid (2–4 mg./g.) in well-fermented cigar tobacco leaves exceeds, by a factor of about 20, the amounts found in other plant materials.[2]

The uptake of considerable quantities of oxygen during the fermentation makes it likely that we have to deal here with a direct or indirect autoxidation of the alkaloid at the relatively low temperatures of the fermentation process (45–55°C.). The question whether this easy oxidation is due to the catalytic action of relatively simple leaf components, or to enzymes contained in the leaves or in a bacterial flora developing on the leaves remains open. However, the isolation of this and other clearly defined reactions as characteristic effects of the tobacco fermentation may help to answer this question after further investigations.

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[1] Identification by melting point, specific ultraviolet absorption, bioassay. For the latter, the authors are greatly obliged to Professor Ray F. Dawson, Columbia University.

[2] A survey of 18 publications showed nicotinic acid contents, ranging from 0.01 to 0.25 mg./g. of dry weight, for various vegetable materials including those richest in the acid, such as wheat, bran, tea, water cress, *etc.*

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Isolation of Crystalline Trehalose after Fermentation of Glucose by Maceration Juice [*]

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INTRODUCTION

The comparatively rare sugar trehalose frequently occurs in bakers' yeast. In 1925 Koch and Koch (1) prepared small amounts of crystalline trehalose by alcohol extraction of yeast treated with ether. Kluyver and van Roosmalen (2) mention that the sugar was isolated in 1929 by van Gent from Delft yeast, and they could themselves prepare about 0.3% of the sugar from the same yeast. Steiner and Cori (3) found 0.5–1.5% trehalose in fresh yeast. The yield was increased after fermentation of glucose. Myrbäck and Örtenblad (4, 5) showed that Swedish bakers' yeast regularly contains relatively large amounts of the sugar, in some cases up to 15% of the dry weight. A convenient method to prepare the sugar from bakers' yeast was also indicated. Tanret (6) found up to 20 g. trehalose/kg. yeast. Müntz (7) has pointed out that, in contrast to bakers' yeast, brewers' yeast (bottom yeast) contains no trehalose. This result was confirmed by Tanret (8) and by Myrbäck (5).[1]

The formation of a trehalose monophosphoric acid during fermentation of glucose (or fructose) by dried yeast and yeast juice was discovered by Robison and Morgan (10, 11) in 1928.

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[*] By agreement with Professor Ragnar Nilsson, this paper was to have been preceded by a paper by Nilsson and Alm, entitled "On the Role of Adenylpyrophosphatase in Alcoholic Fermentation, and on the Occurrence of Trehalose during Fermentation with Maceration Juice," with which it is connected and to which reference is made in the text. I received the paper by Nilsson and Alm on July 21, 1948, for publication in the *Archives of Biochemistry.* In consequence of certain proposals by the Editors, with which the authors, for various reasons, could not comply, the paper by Nilsson and Alm will be published in the *Acta Chemica Scandinavica,* 1949.

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[1] In this connection, an observation by Fink and Just (9) is worth noting. They found that *Torula utilis,* which has grown on wood sugar solutions, contains dulcitol, which could be isolated by application of the method worked out by Myrbäck and Örtenblad for trehalose.

Veibel (12) was able to show that the same ester was formed also by fresh brewers' yeast in the presence of toluene. While Kluyver and van Roosmalen (2) prefer the idea that the ester may be formed by direct esterification of preformed trehalose, Veibel (12, 13) is of the opinion that the trehalose monophosphoric acid originates from glucose, possibly by a synthesis from glucose monophosphoric acid + glucose.

When glucose is fermented by maceration juice (Lebedew juice) prepared from brewers' yeast the total CO_2 evolved is much lower than the theoretical if all the reducing sugar had disappeared through fermentation. If the initial molar concentration of phosphate is equal to, or higher than, that of the sugar, the deficit mainly corresponds to hexose diphosphate. This is not possible, as Nilsson (14) shows, when the total concentration of phosphate is lower than that of the sugar. The circumstances under which this "carbon dioxide deficit" occurs have been thoroughly investigated by Nilsson and coworkers (paper by Nilsson and Alm mentioned in footnote) in a series of papers on sugar fermentation by different yeast preparations. In this case the cause of the CO_2 deficit is the formation from glucose of some nonfermentable substance. In 1936 Sobotka and Holzman (15) found that the deficit corresponded to the appearance of a nonreducing substance (or mixture of substances) which shows an exceptionally high *dextro* rotation. The behavior of the substance(s) on acid hydrolysis intimated the presence of trehalose or some trehalose phosphoric acid. Up to a certain point, the same result was reported by Nilsson (16), who found that the substance was nonreducing, free from phosphorus and, on acid hydrolysis, gave a reducing sugar.

During investigations on the action of arsenate on yeast fermentation we had ample opportunity to observe the CO_2 deficit. The fact that the unknown substance, corresponding to the deficit, is not fermented in the presence of arsenate proves, of course, that it cannot be identical with fructose diphosphoric acid as Meyerhof (17) supposes. As we did not know that Nilsson and Alm continued their investigations, we took up the question of the nature of the unfermentable substance formed during the fermentation in maceration juice. As we also found that this substance yielded reducing sugar by acid hydrolysis and as Sobotka actually had tentatively proposed the trehalose idea, it seemed worth while to try the isolation of trehalose by the method developed for the isolation of the sugar from bakers' yeast.

EXPERIMENTAL

The fermentation experiments were made with maceration juice prepared according to Nilsson (18). Three different preparations of dried yeast were employed (I, II, III). In the experiments with I and II the yeast was macerated for 2 hr. at 38–40°C.; with yeast III, the time of maceration was extended to 2.5 and 3 hr. Equal volumes of maceration juice and 10% glucose solution were mixed and one drop of a 2% acetaldehyde solution/ml. maceration juice added. The amount of CO_2 evolved was measured volumetrically over mercury. When the gas evolution had ceased (after

4–6 hr.) the fermentation mixtures were diluted with 4–5 parts of water, and the proteins, *etc.*, precipitated with mercuric sulfate in H_2SO_4 according to West, Scharles and Peterson (19). The filtrate was neutralized with $BaCO_3$, and the precipitate filtered off and thoroughly washed with water. Filtrate and washings were acidified with H_2SO_4, mercury was precipitated with H_2S and the filtrate aerated. Determinations of phosphorus showed that about 80% of the phosphorus compounds were eliminated. Most of the remaining phosphoric esters were removed by precipitation with barium acetate and $Ba(OH)_2$ in 50% alcohol. The filtrate was concentrated to about $\frac{1}{10}$ volume. With this solution, freed from proteins, barium ions, and the major part of the phosphoric esters, the following determinations were made: (1) reducing substances calculated as glucose (Bertrand method); (2) total reducing substances after 4 hr. hydrolysis with *N* sulfuric acid in a water bath; (3) optical rotation. The amount of trehalose present was tentatively calculated on the assumption that the optical rotation is due to trehalose only.

TABLE I

The Formation of Trehalose from Glucose in Maceration Juice

No.	Yeast	Glucose	Rotation in 2 dm. tube	Trehalose + $2H_2O$ mg., calculated from the optical rotation	Trehalose as glucose	Trehalose in percent of substrate	Reducing substance after fermentation		Difference in mg.	Correction for incomplete hydrolysis	Difference in per cent of substrate
							Before hydrolysis	After hydrolysis			
		mg.			*mg.*						
1	I	0	0	0	0	0	0	0	0	0	0
2	I	3000	2.19	614	584	19.5	66	567	501	588	19.6
3	I	3000	2.24	629	599	20.0	—	—	—	—	—
4	I	3200	2.53	711	677	21.1	—	—	—	—	—
5	I	3000	2.42	679	647	21.5	—	—	—	—	—
6	II	2900	1.60	449	428	14.7	212	480	268	315	10.9
7	II	3000	1.67	469	447	14.9	315	535	220	259	8.6
8	III	3200	1.93	542	515	16.1	118	421	303	356	11.1
9	III	3100[a]	2.14	604	572	18.5	156	603 637[c]	447	526	17.0
10	III	3100	1.77	497	473	15.3	148	458	310	364	11.7
11	III	3000[b]	2.02	564	537	17.9	—	—	—	—	—
12	III	1000[a]	0.68	192	183	18.3	48	214[d]	166	177	17.7

[a] Maceration time 2.5 hr.
[b] Maceration time 3 hr.
[c] Hydrolysis during 5 hr.
[d] Hydrolysis during 6 hr.

From Table I we see that

(1) in Exp. 1, in which maceration juice without addition of glucose is used, no *dextro*-rotatory substance is formed. No reducing sugar is found after acid hydrolysis;

(2) in the final solutions a certain amount of reducing substances is present, varying from 2 to 10% of the added glucose. The lowest value is found in Exp. 2 where arsenate is present, the highest in Exps. 6 and 7 with yeast II, which has an exceptionally low fermenting power;

(3) acid hydrolysis produces large amounts of reducing sugar;

(4) that the final solutions are strongly *dextro*-rotatory.

These results are quite in agreement with those of Sobotka and (concerning points 1–3) with those of Nilsson. They seem to indicate that trehalose is present. However, we must not overlook the possibility that glycogen could be synthesized during the fermentation. But, in our experiments, glycogen should have been precipitated along with the phosphoric esters. Our solutions gave no color with iodine.

In the solution from Exp. 2, combined phosphorus was determined according to Embden; 12.8% of the total phosphorus of the maceration juice was present. This may possibly be due to the higher solubility of the barium salt of trehalose phosphoric acid in diluted alcohol. That is why it is quite possible that, in the solutions in question, trehalose phosphoric acid is present. In this paper, however, we restrict ourselves to the occurrence of trehalose itself.

We applied the method of Myrbäck and Örtenblad on the solution from Exp. 4. After the proteins and the major part of the phosphoric esters had been removed, as we described above, the alcohol was distilled off, the barium ions were precipitated with H_2SO_4 and the filtrate concentrated under reduced pressure to a sirup. A few ml. of water were added and the solution was brought to 35°C. Alcohol at the same temperature was added. The final alcohol concentration was 88%. The solution became slightly opalescent but nothing could be removed by filtration. After a few days in the ice chest crystallization began. The rate of crystallization could be accelerated by seeding a few trehalose crystals. After 10 days the crystals were collected, washed with alcohol, and dried in a vacuum desiccator. The yield was 335 mg. or 14.3% of the glucose added originally. M.p. 95.3°C. Myrbäck (5) found, for pure trehalose, m. p. 96°C.

In Exp. 5 the weight of the crystals was 383 mg., or 17% of the glucose. After recrystallization the m.p. was 96.2°C. and $[\alpha]_D^{20} = 180.1°$. Schukow (20) found $[\alpha]_D^{20} = 178.3°$ for trehalose with $2H_2O$. The water of crystallization, determined after drying for 24 hr. at

125°C., was 9.55%. The theoretical value for trehalose with $2H_2O$ is 9.53%. The form of our crystals was that of pure trehalose.

In Exp. 11 an attempt was made to isolate the trehalose without precipitation of the phosphoric esters, but in this case the trehalose crystals were mixed with a sticky substance, possibly trehalose phosphoric acid.

From Table I we see that, in all experiments, the amount of trehalose calculated from the optical rotation is notably higher than that derived from the hydrolysis. This is the fact even when the nonfermented glucose, determined as directly reducing substance, is subtracted. Different causes may explain this difference. One is that the trehalose is incompletely hydrolyzed after 4 hr. in *N* H_2SO_4, another is that the trehalose is mixed with its phosphoric ester which, according to Robison and Morgan (10), is hydrolyzed more slowly than the sugar itself.

Winterstein (21) found that 5–6 hr. of hydrolysis in *N* H_2SO_4 at 100°C. is required to achieve a nearly complete splitting of the sugar. Steiner and Cori (3) calculated that the degree of hydrolysis in *N* H_2SO_4 is 95–98% after boiling the sugar in the water bath for 8 hr.

We dissolved 164.4 mg. of recrystallized trehalose, m.p. 96.2°C., in 20 ml. of *N* H_2SO_4 and boiled the solution under reflux in the water bath. After 4 and 6 hr., samples were taken out and neutralized. The reducing substance formed was determined according to Bertrand. The degrees of hydrolysis proved to be 85 and 93%, respectively. A determination was also made according to Blom and Rosted (22) and the same values obtained.

DISCUSSION

From the experiments mentioned we see that our values of trehalose in Table I, derived from the hydrolysis, are at least 15% too low. The yield of the reducing substance in Exp. 9 is 7.6% higher if the hydrolysis is extended from 4 to 5 hr. It thus confirms our calculations, and is the reason for introducing a correction of the reducing substance into the table. There is then a good agreement between the amount of trehalose derived from the optical rotation and from the hydrolysis in experiments with yeast I (Exp. 2) and with yeast III when the time of maceration is extended from 2 to 2.5 and 3 hrs. (Exps. 9 and 11). With yeast II, which had a very low fermenting power, and with III after 2 hr. maceration, there is still a difference in the values.

Exps. 9, 11, and 12 tend to show that the yield of trehalose is increased when the time of maceration is increased. This is of interest in connection with the stability of the enzymes concerned with trehalose fermentation. Trehalose added to fresh maceration juice is readily fermented, but, if the disaccharide is added to fermenting mixtures of glucose where the evolution of CO_2 has ceased (at about 4–5 hr. fermentation in experiments without addition of phosphate), the further fermentation of trehalose is very much slower than in the fresh juice. As Myrbäck and Örtenblad (23) have pointed out, the enzymes necessary for trehalose fermentation in Lebedew juice show a very low stability and it can be assumed that these enzymes have been at least partly destroyed during the experiments. This explains also the increased yield of trehalose in juices after prolonged maceration.

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SUMMARY

When glucose is fermented by maceration juice (Lebedew juice) from brewers' yeast a "carbon dioxide deficit" is observed which amounts to about 45% of the added sugar. This deficit corresponds to the formation of strongly *dextro*-rotatory nonreducing substances, yielding glucose upon acid hydrolysis. From such mixtures pure trehalose has been isolated in the crystalline state. The yield was in one case 32%, in another 38%, of the deficit. The occurrence of trehalose phosphate which may be the primary product of a synthesis from glucose phosphate and glucose seems probable.

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Studies on the Methane Fermentation. VII. Tracer Experiments on the Mechanism of Methane Formation[1]

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INTRODUCTION

There is much evidence in support of the theory proposed by van Niel that methane is formed biologically by reduction of carbon dioxide (1). In the methane fermentation of several substrates, such as hydrogen (2, 3, 4, 5, 6), primary and secondary alcohols (1, 7, 8), and some fatty acids (1, 12), it has been shown that the quantity of CO_2 utilized is approximately equivalent to the methane formed. Tracer experiments with C^{11} have also demonstrated that at least some of the methane formed in the fermentation of methyl alcohol is derived from CO_2 (9). Until recently, all the available evidence was consistent with the CO_2 reduction theory of the origin of methane. No other theory has been suggested that even attempts to provide a general explanation for the formation of methane from the many substrates that can be decomposed by methane-producing bacteria. ۱۹۴۴ ✓

In view of this situation, the results recently reported by Buswell and Sollo (10) were entirely unexpected. Buswell and Sollo used C^{14} to investigate the role of CO_2 in the fermentation of acetate by enrichment cultures. When acetate was decomposed in the presence of labeled CO_2 relatively little of the isotope was recovered in the resulting methane. The specific activity of the methane collected two days after the start of the experiment was approximately 1/200 that of the CO_2; after 12 days, the activity of the methane had risen to 1/20 that of the CO_2. From these observations, it was concluded that the methane was produced mainly from acetic acid and only to a slight extent from CO_2.

Since these conclusions appeared to be at variance with all previous evidence concerning the mechanism of methane formation, it seemed desirable to repeat and extend the experiments of Buswell and Sollo. Therefore, we have studied methane fermentations of acetate and ethyl alcohol by tracer methods, using C^{14}-labeled CO_2 and acetic acid.

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MATERIALS AND METHODS

In the acetate fermentations, partially purified cultures of methane-producing bacteria were used because pure cultures capable of attacking acetate were not available. The bacteria in these cultures were mainly micrococci occurring in zoogloeal masses. They probably belong in the genus *Methanococcus*. At no time were any cells of the acetate-fermenting *Methanosarcina* observed.

The bacteria were grown anaerobically in a synthetic mineral medium (1) containing 3–4 m*M* of sodium acetate and 3–5 m*M* of $NaHCO_3$ in a total volume of 100 ml. When active fermentation had started the C^{14}-labeled substrate was added. Oxygen was removed from the gas over the medium by placing a piece of absorbent cotton saturated with a solution of a chromous salt in the neck of the flask. The evolved gases were collected over mercury using an all-glass apparatus. In each experiment the fermentation gases were collected in 2 or 3 fractions that were analyzed separately. Samples of the medium were withdrawn for analysis immediately after addition of the radioactive substrate and after fermentation was complete.

For the alcohol fermentation, a pure culture of *Methanobacterium omelianskii* was grown in 100 ml. of an ethanol-bicarbonate medium (7) to which was added sterile C^{14}-labeled Na_2CO_3. The collection of gases and analyses were done as in the acetate experiments. In addition, the activity of the thoroughly washed cells from an aliquot of the medium was determined.

Acetic acid was determined by titration, following steam distillation in the apparatus described by Markham (13), and was checked for purity by Duclaux distillation. Ethyl alcohol was determined by the method of Widmark (14). Dissolved CO_2 was estimated manometrically (15) and was recovered as $BaCO_3$. Gaseous CO_2 was measured by absorption in alkali. Methane was combusted by passing it over hot CuO containing 1% iron oxide (16); the resulting CO_2 was absorbed in alkali. Dissolved methane was estimated from solubility data. The radioactivity of the acetate and CO_2 was determined on the barium salts by means of a Geiger-Mueller counter.

EXPERIMENTAL RESULTS

Fermentation of CH_3COOH and $C^{14}O_2$

The fermentation of acetate in the presence of C^{14}-labeled CO_2 gave essentially the same results (Table I) as were obtained by Buswell and Sollo (10). The specific activity of the evolved methane was much less than that of the CO_2. When approximately ⅓ of the acetate had been fermented, the ratio of the specific activity of methane to that of the CO_2 was 0.033. Near the end of the fermentation, when most of the acetate was decomposed, the ratio had risen to 0.107.

The data of Table I show that only a small part of the methane was derived from labeled CO_2 added to the medium. This result cannot be due to the failure of the labeled CO_2 to enter the cells, because a very similar yield of labeled methane was obtained when labeled CO_2 was produced inside the cells by the decomposition of carboxyl-labeled acetate (see below). Since the experiment was performed with an impure culture, it cannot be concluded with certainty that the labeled methane was formed by reduction of CO_2 by the methane bacteria. It is possible that other anaerobic bacteria used CO_2 to synthesize labeled acetic acid or other fermentable compounds, which were then decomposed by methane bacteria to give labeled methane. Some support for this possibility was given by the observation that the volatile acid present in the medium at the end of the fermentation contained a small amount of C^{14} (1170 cts./min.). However, it seems more probable that the labeled methane was formed by direct reduction of CO_2 in this experiment.

TABLE I

Specific Activities of Carbon Dioxide and Methane Formed in the Fermentation of $CH_3COOH + C^{14}O_2$[a]

Incubation time	Volume of gas sample	Specific activities, cts./min./m*M*	
		CO_2	CH_4
days	*ml.*		
0		15,700[b]	
18	62	8,670	290
35	74	4,630	290
65	71	3,640	390
65	Dissolved gas	3,550	

[a] 115% of the added C^{14} was recovered in the products.

[b] Specific activity of 2.69 m*M* CO_2 present in the medium initially.

The decrease in specific activity of the evolved CO_2, shown in Table I, was due to the formation of unlabeled CO_2 from acetate. The observed specific activity of the CO_2 in each gas sample agreed within a few per cent with the calculated value, assuming that one mole of CO_2 was formed per mole of methane. The observed values were generally a little lower than the calculated values. This is undoubtedly attributable to the fact that the actual yield of carbon dioxide was always a little greater, and the yield of methane a little smaller, than one mole per mole of acetate decomposed.

Fermentation of $C^{14}H_3COOH$

When methyl-labeled acetate was fermented in the presence of unlabeled CO_2, the evolved methane contained most of the C^{14}, whereas the CO_2 was only feebly radioactive (Table II). A comparison of the specific activities of the added acetate and the methane shows that in the first part of the fermentation at least 94% of the methane must have been derived from the acetate methyl group. In the second part of the fermentation at least 86% of the methane must have come from this source. The decrease could be due either to a direct reduction of inactive CO_2 to methane, or to a dilution of the labeled acetate with inactive acetate synthesized by other organisms in the culture. The specific activity of the evolved CO_2 (after correcting for isotopic dilution by bicarbonate added to the medium) is such as to indicate that from 2.8 to 3.5% of it was derived from the acetate methyl group.

TABLE II

Specific Activities of Carbon Dioxide and Methane Formed in the Fermentation of $C^{14}H_3COOH$ [a]

Incubation time	Volume of gas sample	Specific activities, cts./min./m*M*		
		Substrate added [b]	CO_2 [c]	CH_4
days	*ml.*			
0		52,500		
5	39		300	49,300
20	58		700	45,300
20	Dissolved gas		890	

[a] 103% of the added C^{14} was recovered in the products.

[b] 3.90 m*M* of labeled acetic acid were present initially; 3.88 m*M* were decomposed during the experiment

[c] 5.49 m*M* of unlabeled CO_2 were present initially in the medium.

Fermentation of $CH_3C^{14}OOH$

The C^{14} from carboxyl-labeled acetate went mainly into the evolved CO_2 (Table III). The specific activity of the CO_2 increased during the

TABLE III

Specific Activities of Carbon Dioxide and Methane Formed in the Fermentation of $CH_3C^{14}OOH$ [a]

Incubation time	Volume of gas sample	Specific activities, cts./min./mM		
		Substrate added [b]	CO_2 [c]	CH_4
days	*ml.*			
0		18,700		
10	47		4,530	160
20	40		6,500	850
20	Dissolved gas		7,000	

[a] 82% of the added C^{14} was recovered in the products.

[b] 3.33 mM of labeled acetic acid were present initially; 3.30 mM were decomposed during the experiment.

[c] 5.27 mM of unlabeled CO_2 were present initially in the medium.

fermentation as more of the carboxyl carbon was converted to CO_2. The observed specific activities after 10 and 20 days incubation agreed rather closely (±5%) with the values calculated on the assumption that all the evolved CO_2 was derived from the acetate carboxyl group. In this connection it should be noted that the previous experiment with methyl-labeled acetate demonstrated that a small fraction of the CO_2 was derived from the methyl group.

The small amount of C^{14} in the methane might have been derived either directly from the carboxyl group or from labeled CO_2 that was formed as soon as the fermentation started. However, a comparison of the data in Tables I and III indicates that CO_2 was the main source. The expected specific activity of the methane at the end of Expt. 3, based on the specific activity of the CO_2 at the end of Expt. 3 and the observed ratio of the specific activities of methane and CO_2 in the latter part of Expt. 1, is 6500 cts./min./mM × 0.107 = 695 cts./min./mM. This does not differ significantly from the observed value of 850 cts./min./mM. It must be concluded that very little, if any, methane was derived directly from the acetate carboxyl group.

Fermentation of CH_3CH_2OH and $C^{14}O_2$

The substrate concentrations in this experiment were so chosen that ethyl alcohol was the limiting compound and CO_2 was present in excess. Therefore, at the end of the fermentation the alcohol had disappeared entirely, whereas a considerable amount of CO_2 remained in the medium.

The data in Table IV show that, in the ethanol fermentation, methane was derived mainly if not entirely from CO_2. A comparison of the specific activities of the methane and CO_2 indicates that from 82 to 100% of the methane came from this source. The uncertainty in this value arises from the fact that there was some discrepancy among the 3 estimations of the specific activity of the CO_2. A small amount of C^{14}, corresponding to approximately 3% of the CO_2 reduced, was found in the bacterial cells. This is undoubtedly a minimum value, since some of the soluble constituents of the cells were probably lost during the washing required to remove labeled CO_2. The acetic acid formed during the fermentation was found to be completely inactive. Qualitatively, these results are in accord with those obtained previously by the use of C^{11} (9).

DISCUSSION

Buswell and Sollo (10) have shown that the methane formed by the fermentation of acetic acid in the presence of C^{14}-labeled CO_2 is only slightly labeled. We have confirmed this observation and shown that the methane is derived mainly (86–94%) from the methyl group of the acetic acid, whereas the CO_2 is derived mainly (96–98%) from the carboxyl group. A small part (3–11%) of the methane, which increases as the fermentation progresses, is derived from CO_2. It is probable, in

TABLE IV

Specific Activities of Carbon Dioxide and Methane Formed in the Fermentation of C_2H_5OH + $C^{14}O_2$ by Methanobacterium omelianskii[a]

Incubation time	Volume of gas sample	Specific activities, cts./min./m*M*	
		CO_2	CH_4
days	*ml.*		
0		27,000[b]	
15	84	31,900	26,200
25	27	34,200	28,100
25[c]	Dissolved gas	33,000	

[a] 112% of the added C^{14} was recovered in the products.

[b] Specific activity of 5.46 m*M* of CO_2 present initially in the medium.

[c] 8.69 m*M* of ethyl alcohol were decomposed during the fermentation.

view of the similarity of our results with those obtained by Buswell and Sollo, that this small conversion of CO_2 to methane is catalyzed by methane-producing bacteria. However, since both series of experiments were carried out with impure cultures, the possibility that the initial utilization of CO_2 was due to other anaerobic bacteria, perhaps similar to Wieringa's *Clostridium aceticum* (11), cannot be excluded entirely.

The results obtained in the fermentation of ethyl alcohol by a pure culture of *M. omelianskii* are equally conclusive in showing that the methane was derived almost entirely (82–100%) from CO_2. Furthermore, neither ethyl alcohol nor acetic acid can have been an intermediate in this process, since the residual acetic acid, formed in the presence of labeled CO_2, was completely non-radioactive.

It must be concluded that methane can be formed from at least two sources, CO_2 and the methyl group of acetic acid. Various lines of evidence, referred to in the introduction, indicate that CO_2 is more commonly used, although thus far the conversion of CO_2 to methane has been proven conclusively with only a few substrates.

The known facts concerning the origin of methane in the fermentation of acetic acid can be interpreted in different ways. Buswell and Sollo apparently consider a simple decarboxylation to be the most likely mechanism. This is consistent with the experimental facts, but appears to us to be unacceptable from a biochemical point of view. Several decarboxylation reactions are known to be catalyzed by microorganisms, but not one of them is capable of supplying energy needed for growth and other biological processes. In fact, it seems unlikely that useful chemical energy can be obtained by living organisms from any process, such as a simple decarboxylation, that consists only of a rearrangement of the atoms within a single molecule.

Since all the known biochemical processes that support growth of microorganisms involve oxidation-reduction reactions, it seems safe to conclude that the same is true of the fermentation of acetic acid, which supports the growth of the methane-producing bacteria. If this is accepted, it follows that the fermentation is a complex process in which acetic acid is oxidized in such a way as to liberate its carboxyl groups as CO_2 while the methyl group is converted to some compound (not necessarily a C_1 compound) that can be reduced to methane.

The concept that the acetate fermentation is a complex process consisting of many steps makes it possible to fit the results of the tracer experiments into the CO_2 reduction theory. It is essential to do this because we know that some acetate-fermenting methane bacteria are able to reduce CO_2 to methane, using hydrogen as a reductant (3, 4), and it is unlikely *a priori* that one organism would possess two entirely different mechanisms for making methane. The simplest way of combining the known facts into a unified theory is to assume that there is a common intermediate (X) in the conversion of both CO_2 and acetic acid to methane, as indicated in the following scheme:

$$\begin{tikzcd}
CH_3COOH \arrow[dr] & & \\
& X \arrow[r] & CH_4. \\
CO_2 \arrow[ur] & &
\end{tikzcd}$$

This implies that the final steps in the formation of methane are the same with both substrates and only the early steps differ. To explain the small CO_2 utilization during acetate fermentation, it must be assumed further that the formation of compound X from CO_2 is prevented, or greatly retarded, by a product of acetate decomposition. This inhibition could be due to competition between intermediates of similar structure or to other causes. It cannot be due to the action of acetate itself because acetate does not interfere with CO_2 utilization by *M. omelianskii* and other bacteria (12) that are unable to ferment it.

The assumption that the utilization of CO_2 is suppressed by products of acetate breakdown receives some possible support from the observation that the fraction of methane derived from CO_2 increases near the end of the fermentation when the acetate concentration is relatively low. However, as has already been pointed out, this phenomenon may also be interpreted in other ways.

SUMMARY

By using C^{14}-labeled carbon dioxide and acetic acid it has been shown that, in the methane fermentation of acetic acid, the methane is derived mainly from the methyl group, whereas the CO_2 is derived from the carboxyl group. A little CO_2 is converted to methane, the amount increasing toward the end of the fermentation.

The methane formed in the fermentation of ethyl alcohol by *Methanobacterium omelianskii* is derived from CO_2 and not from the alcohol or acetic acid.

The significance of the above results in relation to the CO_2 reduction theory of methane formation is discussed.

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Transmethylation of Guanidoacetic Acid in Beef Liver Autolyzates

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INTRODUCTION

Following studies on the transmethylation of guanidoacetic acid in liver and kidney slices (1), Borsook and Dubnoff studied the conditions for creatine synthesis from guanidoacetic acid and methionine in guinea pig liver homogenates (2). An investigation was undertaken in this laboratory with a view to obtaining an enzyme preparation from liver which would have high creatine-synthesizing activity and which would contain in solution all the enzyme available in the source from which it was made. Such a preparation would be useful in the further elucidation of the mechanism of the transfer of the labile methyl group of methionine to guanidoacetic acid, and of the roles of adenosine triphosphate (ATP) and of oxidative processes in the formation of creatine.

A number of techniques were used in attempting to get active products. When beef liver blends (Waring Blendor) were fractionated by centrifugation (3500 r.p.m.) into supernatant and washed precipitate (using $M/10$ neutral phosphate buffer), both fractions were found to possess enzyme activity. A desiccated and defatted beef liver powder (using successively acetone, acetone-ether and ether extraction in the cold) possessed creatine-synthesizing activity, but also failed to possess enzyme activity predominantly in either one or the other fraction, whether the extractant was water, neutral phosphate buffer, M NaCl, or physiological saline. The results with these two types of liver preparation suggested that, while the enzyme system responsible for the transmethylation of guanidoacetic acid is water-soluble, these methods cannot wholly liberate the system from remaining intact cells or from some water-insoluble fraction of the liver cell. This preliminary work will not be described further.

A third type of preparation, a liver autolyzate, gave encouraging results. Virtually all the active material of the liver was found in the water-soluble fraction of the autolyzate. Some properties of the transmethylating enzyme system studied in autolyzates are described below.

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EXPERIMENTAL

Liver autolyzates were prepared by blending 100 g. of beef liver, 100 ml. of ice-cold water and 20 ml. of toluene in a Waring Blendor. To prevent the mixture from warming up too much during the blending, some of the water was replaced as necessary by cracked ice. The blended liver was allowed to autolyze at 20°C. for 20 hr. It was found that this period is sufficient to render most of the tissue components soluble in water, yielding a solution having considerable creatine-synthesizing activity. An initial study of the effect of temperature of autolysis on the activity of the preparation (see "RESULTS") led to the selection of autolysis at 20°C. in all further work.

The autolyzate was diluted with 50 ml. of water and centrifuged at 1500 r.p.m. in a refrigerated centrifuge for 30 min. The supernatant was decanted through two layers of cheese-cloth into a large beaker, any residue on the cloth being returned to the loose residue in the centrifuge bottle. The residue was washed twice more. This was generally sufficient to extract the major portion of the soluble materials. The supernatant and washings were finally combined, yielding 3–5 ml. of solution/g. of liver taken. The insoluble portion of the autolyzate consisted essentially of the peritoneal covering of the liver, blood vessel walls, and connective tissue.

Activity was tested as follows: One ml. of the liver preparation was added to a reaction mixture containing guanidoacetic acid (0.001 *M*), DL-methionine (0.05 *M*), ATP (0.0015 *M*) and phosphate buffer, pH 7.4 (0.025 *M*), with a final volume of 4.0 ml. The ATP was prepared by the method of Dounce and coworkers (3); analysis of one batch of the barium salt of this ATP gave: total P, 10.48% (theor. 10.88%); inorganic P, 1.5% (theor. 0.00%); P hydrolyzed at 100°C. in 1 *N* HCl in 7 min., 63.1%, in 15 min., 66.5% of organic P (theor. 66.67%). The author is indebted to Dr. P. S. Krishnan for supplying some of the ATP used in this work.

The reaction mixtures were incubated for 2 hr. in 180 × 25 mm. test tubes supported in a water-bath maintained at 40 ± 0.05°C. At the start of incubation the tubes were vigorously gassed with oxygen to replace the air, and thereafter oxygen was bubbled through the reaction mixtures at a steady rate.

At the end of the incubation period, the reaction was stopped with 0.5 ml. of 30% trichloroacetic acid, which also served to precipitate the proteins. The latter were removed by filtration, and "total Jaffe-reactive substances" in an aliquot of the protein-free filtrate were estimated, as creatine (4), according to the following procedure: A 1 ml. aliquot of the protein-free filtrate was autoclaved with 4.0 ml. of 1% picric acid at 121°C. for 0.5 hr. After cooling the solution to room temperature, 0.3 ml. of 10% NaOH were added and the color allowed to develop for 15–20 min. The solutions were read in a Klett-Summerson photoelectric colorimeter using a standard Corning 540 mμ (green) filter. Density readings were converted to mg. of creatine using a calibration curve. Blanks consisted of the appropriate reaction mixture, including the enzyme preparation, in which the reaction was stopped at "zero" time of incubation with trichloroacetic acid. Each reaction mixture (and the corresponding blank) was carried through the incubation and subsequent procedure in duplicate or triplicate, and these values were averaged.

In the incubated tubes, "total Jaffe-reactive substances" consist of preformed Jaffe-reactive substances, creatine formed during the incubation, and unreacted guanidoacetic acid; in the blanks they consist of preformed Jaffe-reactive substances plus initial guanidoacetic acid. The difference between "incubated" and "blank" values reduces to "formed creatine less an equimolar amount of guanidoacetic acid, estimated as creatine." Basal creatine production in the absence of substrates is negligible. It was found that approximately 10.7 mg. of guanidoacetic acid (standard error, 0.2 mg.) give the same colorimeter reading as 1 mg. of creatine, after these compounds have been converted to their respective cyclic derivatives through autoclaving in acid and treatment with sodium picrate according to the modified Folin method used. From this relation, the "apparent creatine formed" has been calculated to be 92% of the true value (standard error, 3.3%). Since, in this study, we were particularly interested in relative activity of preparations, this constant error was not considered important.

Dry matter in the liver preparations was determined by drying 1 ml. of the preparation to constant weight at 70°C.

RESULTS

Activity of the Soluble and Insoluble Fractions of Beef Liver Autolyzates, and the Effect of Temperature of Autolysis

Beef liver autolyzates prepared at 3 different temperatures, namely, 4, 20 and 37°C., respectively, were fractionated into soluble and insoluble portions as described above. The water-insoluble fraction was made up with water to the same volume as the soluble fraction, and the activity of each was tested. The results are given in Table I.

Autolysis at 20°C. consistently gave more active soluble fractions than when the autolysis proceeded in the cold. At the latter tempera-

TABLE I

The Creatine-Forming Activity of Soluble and Insoluble Fractions of Beef Liver Autolyzates

Temperature of autolysis (°C.)	Amount of creatine formed			
	Micromoles		Micromoles/g. of dry matter	
	Soluble	Insoluble	Soluble	Insoluble
4	0.98	0.17	35.7	30.8
20	1.21	.03	38.7	2.2
37	.34	.00	20.3	.0

ture, substantial activity could be recovered in the insoluble fraction. The 37°-autolyzate yielded a soluble fraction low in activity.

The soluble fraction of the 20°-autolyzate, hereafter referred to as "Autolyzate-Soluble Fraction" (ASF) was selected for further work on the basis of its high activity, and because at this temperature solubilization of the enzyme system appears to be complete. Since aging of the ASF at 4°C. was always accompanied by some loss of activity, a fresh autolyzate was prepared for each experiment day.

The Effect of Partial and Complete Anaerobiosis on Creatine Formation by Autolyzate-Soluble Fraction

Borsook and Dubnoff (2) observed that anaerobiosis is a potent inhibitor of creatine formation in guinea pig liver homogenates. As is seen in Table II, the same is true for the autolyzate preparation. When the atmosphere over the mixture was air, the amount of creatine formed was less than when oxygen was used. The small apparent creatine formation with the nitrogen atmosphere is possibly an artefact due to residual air not completely washed out of the tubes.

TABLE II

Effect of Partial and Complete Anaerobiosis on the Formation of Creatine by ASF

Gas	O_2	Micromoles of creatine formed/g. of dry matter
	per cent	
Nitrogen	0.0	3.3
Air	21.0	20.7
Oxygen	99.5	42.9

The inhibition due to anaerobiosis occurs even in the presence of adequate amounts of ATP, a fact which supports the above authors' conclusion that oxygen is necessary for processes other than the constant replenishment of ATP in the incubated preparation. Moreover, the amount of creatine formed appears to be a function of the oxygen tension in the medium.

Effect of Dialysis of the Autolyzate-Soluble Fraction

When ASF was dialyzed overnight in cellophane bags against a large volume of water at 4°C., there was about 40% decrease in activity. One-third of the Jaffe-reactive substances (estimated as creatine) is lost on dialysis (blank values), as may be seen in Table III.

To determine whether this decrease in activity was due to denaturation of the enzyme system during dialysis or to loss of an activator, the efficacy of adding back dialyzate or a filtrate obtained from boiled ASF to the dialyzed preparation was tested.

The filtrate was prepared by heating ASF on a boiling water-bath for 15 min., then cooling, and filtering off the coagulum. One ml. of the filtrate was included in the standard reaction mixture. The enzyme preparation used was ASF, dialyzed 24 hr. at 4°C. The effect of the boiled filtrate was to decrease the activity of the dialyzed enzyme still further (Table III).

To test the effect of the dialyzate, samples of ASF were dialyzed against an equal volume of water for 4, 8, and 22 hr., respectively. The dialyzed enzyme preparations were then tested alone and with the corresponding dialyzates. Four replicates, whose averages are shown in Table IV, gave essentially the same type of result.

TABLE III

Effect of Filtrate of Boiled ASF on the Activity of the Dialyzed Fraction

Enzyme	Jaffe-reactive substances (as creatine)		Creatine formed
	Incubated	Blank	
	mg.	*mg.*	*mg.*
ASF, stored 24 hr. at 4°C.	0.250	0.156	0.094
ASF, dialyzed 24 hr. at 4°C.	.156	.100	.056
Dialyzed ASF plus boiled ASF filtrate	.221	.187	.034

The results are quite comparable to those observed when the filtrate of boiled ASF was added to the dialyzed enzyme preparation. There is a reduced activity following dialysis, which is of the same order whether dialysis is for 4, 8, or 22 hr. Addition of dialyzate causes a further reduction in activity.

The relatively large contribution of Jaffe-reactive substances by the filtrate and dialyzates (as judged from the blank values in Tables III and IV) suggested that one or other of these substances may be responsible for the lowered activity. Accordingly, the activity of dialyzed ASF was tested in the presence of graded amounts of creatine. The results are shown in Table V.

TABLE IV

The Effect of Dialyzate on the Formation of Creatine by Dialyzed ASF

Enzyme	Jaffe-reactive substances (as creatine)		Creatine formed
	Incubated	Blank	
	mg.	*mg.*	*mg.*
1. ASF	0.321	0.184	0.137
2. ASF, dialyzed 4 hr.	.242	.146	.096
3. (2) plus dialyzate	.254	.193	.061
4. ASF, dialyzed 8 hr.	.228	.140	.088
5. (4) plus dialyzate	.254	.186	.068
6. ASF, dialyzed 22 hr.	.218	.126	.092
7. (6) plus dialyzate	.247	.175	.072

It is seen that creatine added to the reaction mixtures at the start of incubation reduced the activity of the dialyzed ASF by 20–35%. This result may be correlated with the recent evidence of Hoberman, Sims and Peters (5) that the synthesis of creatine *in vivo* is retarded by, or in the presence of, exogenous creatine. While they postulated that creatine is phosphorylated to phosphocreatine, thus reducing the effective concentration of ATP available for transmethylation, it may also be suggested that creatine acts by competition with guanidoacetic acid for the active centers of the enzyme.

The effect of dialysis, therefore, appears to be an average of two processes working in opposite directions: (1) denaturation, decreasing the effective amount of the enzyme, and tending to lower activity; and (2) removal of creatine, an inhibitor of the enzyme, by diffusion through

TABLE V

Effect of Added Creatine on the Formation of Creatine by Dialyzed ASF

Creatine added	Jaffe-reactive substances (as creatine)		Creatine formed	Per cent of original activity
	Incubated	Blank		
mg.	*mg.*	*mg.*	*mg.*	
0.000	0.196	0.100	0.096	(100)
.025	.196	.124	.072	75
.050	.231	.155	.076	79
.100	.256	.194	.062	65

the dialysis bag, tending to increase activity. The first process is quantitatively the more significant.

Effect of Boiled Liver Juice

In contrast to the results described above, boiled liver juice consistently had an activating effect. The juice was prepared by heating 5 g. of minced beef liver together with 20 ml. of water on a boiling water-bath for 15 min., cooling, and filtering. One ml. of the filtrate was included in the the reaction mixture containing the ASF. The boiled liver juice "activated" both dialyzed and nondialyzed ASF (Table VI).

TABLE VI

Effect of Boiled Liver Juice on the Formation of Creatine by Nondialyzed and Dialyzed ASF

(All values are expressed as micromoles of creatine formed/g. of nondialyzable dry matter.)

Boiled liver juice added	Enzyme preparation	
	Nondialyzed ASF	Dialyzed ASF
ml.		
0.0	14.6	10.5
.1	14.8	11.2
.5	16.2	13.8
1.0	18.5	16.3

To exclude the possibility that bile salts play an activating role in the liver juice, sodium taurocholate was added to the reaction mixtures in concentrations up to 2.5 mg./ml. With both dialyzed and nondialyzed ASF, sodium taurocholate inhibited activity, more or less in proportion to its concentration.

It is possible that the Jaffe-reactive substances in the boiled liver juice included guanidoacetic acid in sufficient concentration to serve as an extra substrate, and thereby lead to greater yields of creatine. The loss of this "activator" in the course of autolysis would then be due to its conversion to creatine, which acts as an inhibitor.

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The author is greatly indebted to Professor James B. Sumner for his interest in this work and for advice generously given.

SUMMARY

A liver preparation is described which contains an enzyme system capable of forming creatine from guanidoacetic acid in the presence of

methionine. The preparation, an autolyzed blend of beef liver, consists of water-soluble components; it possesses relatively high creatine-synthesizing activity. Both ATP and oxygen are necessary for the synthesis of creatine.

On dialysis, the autolyzate undergoes some decrease in activity, apparently not due to loss of a cofactor. Creatine added to the reaction mixture inhibits the transmethylation of guanidoacetic acid. Sodium taurocholate also inhibits the reaction. Boiled liver juice has an activating effect.

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Determination of Added Rutin in Urine

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INTRODUCTION

The use of rutin, 5,7,3′,4′-tetrahydroxyflavonol-3-rutinoside, in the treatment of increased capillary fragility sometimes associated with hypertension (1), and in the treatment of certain other pathological conditions related to bleeding and capillary wall strength (2,3,4,5), has been reported in the literature. These clinical and pharmacological studies have indicated the desirability of precise methods for the determination of rutin in urine, and in other biological fluids and tissues. This paper describes a method for the determination of added rutin in urine.

Absorption in the ultraviolet spectrum (6) was not applicable, since interfering materials normally present in urine could not be satisfactorily separated from the rutin. The borocitric complex of Wilson *et al.* (7) and the fluorimetric method of Glazko *et al.* (8), based on the same reaction, were eliminated because of the difficulty of transferring the rutin from the urine into acetone and obtaining the resultant solution in an anhydrous condition.

The $FeCl_3$ reaction was not found useful because of its sensitivity to concentration, pH, and other factors. A search was made for other metal salts which with rutin might produce characteristic absorption in the visible spectrum more favorable to the establishment of a quantitative spectrophotometric method of analysis. Of the twenty-odd salts tested, the most satisfactory was $AlCl_3$, which with rutin produced a relatively stable intense yellow color with a definite absorption maximum at 413 mμ.

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EXPERIMENTAL

Reagents

(a) Aluminum chloride, 0.1 *M*—aqueous solution; (b) ammonium hydroxide, 1 part conc. NH_4OH plus 3 parts water; (c) potassium acetate, 1 *N*—aqueous solution; (d) acetic acid, glacial.

Apparatus

(a) Round-bottom centrifuge tubes, 15 ml.; (b) centrifuge, about 2000 r.p.m. An angle-head type centrifuge did not produce sufficient separation. By changing to a regular clinical centrifuge, the gel could be separated; (c) spectrophotometer or filter photometer, with cell compartment for 5 cm. cells.

Procedure

Preservation and Preparation of Urine Sample. It is convenient to use toluene as the preservative in collecting 24 hr. specimens of urine. However, before the sample for analysis is removed, as much of the toluene as is practical should be removed, so that suspended toluene mechanically carried along by the $Al(OH)_3$ gel does not later produce an error in the spectrophotometric determination. To separate the toluene, about 50 ml. of the 24 hr. specimen is with drawn from the original container by suction through a glass tube having a small orifice. By applying a small vacuum, any emulsion present is broken, and a clear solution is obtained.

Separation of Rutin from the Urinary Pigments. Place 2 ml. of urine in a 15-ml. round-bottom centrifuge tube. Add 3 ml. of 0.1 *M* $AlCl_3$ solution, mix, add 0.5 ml. of NH_4OH (1:3), mix, and dilute to about 10 ml. Centrifuge for 5 min. at 2000 r.p.m. decant the supernatant liquid, and break up the $Al(OH)_3$ gel by briskly tapping the middle of the tube against the finger. Add about 2 ml. of water and continue to break up the gel. Add one drop of the NH_4OH solution, mix, and dilute to about 10 ml. Centrifuge, and repeat the washing procedure. Decant the final wash solution and drain the tube for 20 to 30 sec. Add 0.1 ml. of glacial acetic acid by running it down the side of the tube and break up the gel as before. Stopper the tube and let stand overnight at room temperature to dissolve the gel.

Buffering, Development of Color, and Spectrophotometric Determination. Add 10 ml. of 1 *N* potassium acetate to the solubilized gel, mix, transfer to a 50-ml. volumetric flask, and make to volume with water. After about 30 min., and not more than 2 hr., determine the optical density of the solution at 413–416 mμ in a 5 cm. cell *vs.* a reagent blank carried through the above-described procedure.

Preparation of Standard Curve. Pipette a volume of each standard solution corresponding to 0.020, 0.050, 0.100, 0.150, and 0.200 mg. of the glycoside, into the round-bottom centrifuge tubes. Follow the above-described procedure exactly and plot optical density *vs.* the corresponding weight of rutin taken for analysis.

Calculations. From the standardization curve, determine the weight of rutin in the aliquot of urine taken for analysis, divide by the volume of the aliquot, and report as mg./ml. To report as mg./24 hr., multiply the mg./ml. by the volume for the 24 hr. specimen.

DISCUSSION

The absorption curves for the rutin-aluminum chloride complex and for pure rutin are shown in Fig. 1. The wave length of the maximum is constant if the potassium acetate buffer and 0.1 ml. of glacial acetic acid are used (approximately pH 5.5), but shifts with relatively large changes in pH. Ethanol, if present in relatively large percentages, also shifts the wavelength of the maximum. Aluminum sulfate cannot be substituted for aluminum chloride.

Two to 5 ml. of urine may be used, depending on the concentration of rutin present. With larger volumes of urine, the amount of phosphates precipitated by the reagents is increased. This increased quantity cannot be redissolved in the glacial acetic acid, and a significant error is introduced.

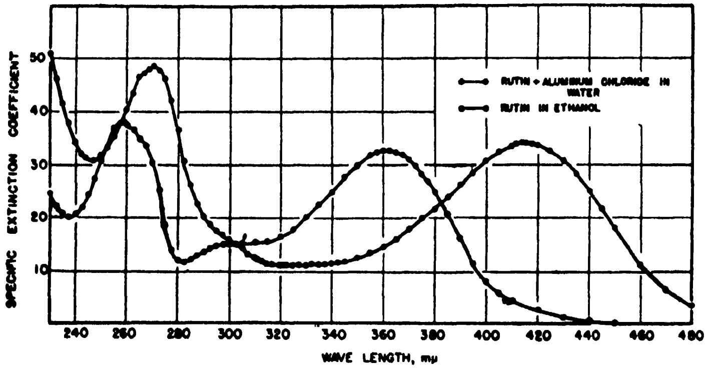

FIG. 1. Absorption spectra for rutin in ethanol and for the rutin-aluminum chloride complex in water. The specific extinction coefficients for both curves are based on 1 g. of rutin/l. in a 1 cm. cell.

Solution of the aluminum hydroxide gel by 0.1 ml. of glacial acetic acid did not appear to be complete at the end of 6 hr. but was complete in 18 hr. Incomplete solution in all samples which had stood for 6 hr. or less was indicated, after addition of the buffer, by a sedimentation of $Al(OH)_3$ containing most of the rutin. Dilute acetic acid did not give complete solution even in 24 hr. Heat decreased the time required for solution, but the results obtained were erratic. Larger volumes of acetic acid lowered the final pH and produced lower optical densities, which could not be raised by further additions of potassium acetate.

Furthermore, the rate of solution was not significantly different even when 5 times as much acetic acid was used.

After addition of the potassium acetate buffer, the color intensity increased for about 30 min., was constant for approximately 3 hr., and then slowly faded over a period of 24 hr. All absorption measurements reported in this paper were made at some time between 30 min. and 1 hr.

The spectrophotometric measurements were made on a General Electric automatic recording spectrophotometer with a reagent blank. A straight line, which passed through the origin, was obtained for the standard curve.

TABLE I

Standardization Data. Rutin-Aluminum Chloride Complex vs. Reagent Blank

Sample	C^a Wt. rutin	D^b Optical density	D/C^c
	mg.		
1	0.223	0.716	3.211
2	.185	.593	3.205
3	.156	.503	3.224
4	.130	.415	3.192
5	.089	.284	3.191
6	.074	.236	3.189
7	.022	.070	3.182
8	.019	.060	3.180

[a] C = mg. of rutin in a 2 ml. aliquot.

[b] D = optical density at 416 mμ, as measured in 5 cm. cells, of a solution containing the weight of rutin shown under "C" diluted to 50 ml.

[c] $10 \times D/C$ = specific extinction coefficient.

The rutin used for these experiments was purified by repeated alternate recrystallizations from absolute ethanol and water. The purified rutin was dried and weighed according to the methods described in a previous paper (6). The dried rutin (25 mg.) was dissolved in 3 ml. of absolute ethanol and diluted to 250 ml. with water. Further dilutions of this solution to obtain the concentrations indicated were also made with water. The analytical data for the standardization are shown in Table I.

In the recovery experiment presented in Table II, the dried rutin was dissolved in 3 ml. of absolute ethanol and diluted to 250 ml. with urine. Subsequent dilutions were made with urine to obtain the desired

TABLE II

Recovery of Rutin Added to Urine

Sample number	Wt. of rutin added to 2 ml. urine A	Total rutin determined per 2 ml. urine B	Added rutin recovered per 2 ml. urine[a] C	Difference $D = C - A$	Average per cent error $\frac{D}{A} \times 100$
	mg.	*mg.*	*mg.*	*mg.*	
Control	0.000	0.007	—	—	—
Control	.000	.007	—	—	—
1	.205	.221	0.214	+0.009	
2	.205	.220	.213	+ .008	+4.2
3	.144	.157	.150	+ .006	
4	.144	.155	.148	+ .004	+3.5
5	.103	.114	.107	+ .004	
6	.103	.114	.107	+ .004	+3.9
7	.041	.050	.043	+ .002	
8	.041	.048	.041	.000	+2.4
9	.021	.027	.020	− .001	
10	.021	.029	.022	+ .001	0.0

[a] The value for added rutin was obtained by subtracting the amount of rutin in the control from the total rutin determined in each sample ($C = B - 0.007$).

concentrations. Recoveries of added rutin have always been within 5% of theory.

To test the applicability of this method to the determination of rutin excreted in urine, a number of experiments were conducted. Four human subjects took by mouth quantitites of rutin ranging from 60 to 2250 mg. a day for 7–14 days. Analyses of the urine excreted by these subjects after variable periods revealed little or no rutin. The quantities detected in 24 hr. samples were so small that they were considered negligible. This was an unexpected finding, especially in view of the reports of Fukuda (9), Fukuda and Kono (10), Kono (11,12), and Garino (13), which indicated a rapid and complete excretion of flavonol glucosides administered to laboratory animals. In some of their experiments rutin was administered intravenously and in considerably larger quantities than were given to our subjects. It is possible that the dose and method of administration may affect the excretion of the glucoside. This problem is being investigated.

Compounds related to rutin, such as quercetin and quercitrin, interfere since they give a similar complex with aluminum chloride having somewhat different absorption curve shapes and maxima.

SUMMARY

A relatively simple method is described for the determination of rutin added to urine. It is based on the color intensity of the complex formed by a buffered solution of rutin and aluminum chloride. Although it is not specific for rutin, the method gives quantitative recoveries of rutin added to urine.

Rutin could not be recovered in more than traces from the urine of 4 human subjects who had taken by mouth quantities of 60–2250 mg. a day for 7–14 days.

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A Quantitative Test of Eggleton's Proposed Mechanism of Nitrogen Loss

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INTRODUCTION

An earlier publication reported extensive nitrogen loss to be an unanticipated and frequent consequence of the culture of excised young shoots of tobacco in solutions of nicotine hydrochloride (1). The circumstances surrounding these losses have since been examined and certain instructive relationships brought to light. Although the results are fragmentary, they appear to be unique in two respects. Firstly, they indicate the conditions under which maximum rates of nitrogen loss might reasonably be expected to occur. Secondly, they permit a quantitative test of Eggleton's original suggestion[1] of chemical mechanism (2). Chibnall (3) has summarized the principal literature on this subject.

Details of experimentation have been reported elsewhere (1). Excised shoots of young tobacco plants (*Nicotiana tabacum* vars. Turkish and Rosenberg) were cultured with their cut ends in solutions of nicotine hydrochloride (0.01 *M*) or of ammonium salts (0.05 *M*). The culture solutions were kept at the temperature of melting ice to avoid interference from microorganisms, while the leafy shoots were exposed to the seasonal day and night temperature and light conditions of the greenhouse without supplementary illumination. At predetermined intervals samples of the cultures were removed and prepared for analysis either by drying in a forced-draft hot-air drying oven at 80°C. or by cold water extraction at the hydraulic press following ether cytolysis. The methods of analysis for the different nitrogen fractions were essentially those of Vickery and his associates (6) as modified in the present author's laboratory for use with small samples (1). Net changes in the total nitrogen of these tissues during culture were measured by suitable modification of the Kjeldahl procedure, using a copper-selenium catalyst and digestion periods that were determined by trial to be adequate for this type of material. Figures so obtained were then compared with the quantities of nitrogen absorbed from the culture solutions as measured by direct analysis. For statistical as well as physiological reasons, the data have been calculated on the basis of absolute amount of nitrogen in mg. per individual shoot. Further breakdown into changes that occurred in stem and leaf as separate entities has been unprofitable due to the occurrence of small but genuinely complicating amounts of translocation.

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[1] $HNO_2 + R\text{-}NH_2 \rightarrow R\text{-}OH + N_2 \nearrow$. Nitrous acid is assumed to arise as an intermediate in the reduction of nitrates in the plant cell (3).

Three experiments, numbered 1, 2, and 3, respectively, were described in the first paper of this series (1). Data from these experiments are discussed in the present paper under the same numerical designations.

Nature and Magnitude of the Nitrogen Losses

Two types of nitrogen loss are recognizable in the present study. In one type (Table I, Expts. 1 and 2) nicotine nitrogen was apparently lost directly from the tissues either as such or, more likely, after chemical modification (*cf.* (1)). The magnitude of such loss was about 25% of the absorbed nicotine nitrogen[2] in each experiment.

TABLE I

Nitrogen Losses from Excised Tobacco Shoots during Culture in Nicotine Hydrochloride Solutions for 96 Hr.

Experiment no.[a]	Time of year	Type of nitrogen loss	
		Alkaloidal	Nonalkaloidal
1	January	15.0	41.0
2	March	16.6 (0)[b]	3.1 (19.7)[b]
3	May	0	0.9

The values in this table are mg. of N per shoot.

[a] Experiments numbered 1, 2, and 3 are identical with Expts. 1, 2, and 3, respectively, of the earlier paper (1).

[b] In this experiment the results permit no unequivocal choice in assigning the total nitrogen loss to one fraction or the other. For inferential reasons (see (1)) it has been assigned to the alkaloidal fraction in larger part (the 3.1 mg. remainder of the nonalkaloidal fraction is statistically non-significant). Alternative figures are given in parentheses.

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[2] Since the pyridine nitrogen of nicotine is known to digest slowly in the Kjeldahl procedure, estimates of total nitrogen of the various tissue samples were controlled by accessory determinations in which nicotine (and ammonia) were removed from alkalinized tissue extracts by steam distillation prior to the conventional Kjeldahl digestion. Summation of the titration values for each of these different fractions yielded figures in close agreement with total nitrogen estimates obtained in the usual manner.

TABLE II

Nitrogen Changes in Excised Tobacco Shoots (var. Rosenberg) during Culture in the Light in Solutions of Ammonium Salts for 96 Hr.

Previously unpublished data from Expt. 3 of (1).

Treatment	Nitrogen fractions						N absorbed	Balance
	Insoluble	Ammonium	Amide	Amino	Nitrate	Undetermined		
Water	+4.29	+1.43	0	+4.71	−14.71	+2.71	0	−1.14
Ammonium chloride	+19.57	+62.00	+16.85	+17.14	−15.00	+19.28	142.00	−22.59[a]
Ammonium nitrate	+18.57	+58.40	+12.72	+22.00	+88.82	+15.57	256.86	−40.64[a]

The values in the table are mg. of N per shoot. Small changes in nicotine nitrogen have been included in the calculation of the balance figures but are not shown in the table. Hence, the figures in the last column do not represent the simple algebraic sums of the figures in the remaining columns.

[a] Computed on basis of amount of nitrogen absorbed from culture solution and regained in tissues by analysis.

The second type of nitrogen loss evidently involved substances other than nicotine and its metabolites (Table I) and occurred extensively only in Expt. 1. This type of loss is at least potentially identifiable with that postulated by Eggleton and forms the subject of the bulk of this paper. The quantities of such nitrogen to disappear during

TABLE III

Absorption of Nicotine and Loss of Nonalkaloidal Nitrogen by Excised Tobacco Shoots (var. Turkish) during Culture in the Light

The data are recalculated from Expt. 1 of the earlier paper (1).

Hr. of culture	Nicotine nitrogen absorbed	Nicotine nitrogen recovered	Nonalkaloidal nitrogen lost	
			Unsmoothed data	Smoothed data
0–24	15.0	9.28	12.00	9.28
24–48	14.64	10.36	7.55	10.36
48–72	16.73	13.72	11.09	13.72
72–96	9.64	7.64	10.91	7.64
0–96	56.01	41.00	41.55	41.00

The values in this table are mg. of N per shoot.

the 96-hr. culture period of Expt. 1 were, within the limits of error, exactly equal to the amounts of nicotine nitrogen absorbed from the culture solution and retained within the tissues (Table III). Thus, since the remainder of the absorbed nicotine nitrogen was lost, the total nitrogen of the successive collections of Expt. 1 (189.0, 186.3, 189.1, 191.7, and 188.5 mg., respectively) showed no consistent or significant change with time. These values for total nitrogen of the successive collections have been smoothed by averaging for use in successive calculations.[3]

Conditioning Factors

In the earlier paper (1) it was shown that about $\frac{1}{4}$ of the absorbed nicotine nitrogen was either lost directly from the shoot tissues (Expts. 1 and 2) or converted into some nonvolatile form which accumulated in the water-soluble nitrogen fraction (Expt. 3). No further evidence is available to indicate the fate of this nitrogen, and the nature of the environmental circumstances which led to its loss in the first two experiments cannot readily be inferred (see below).

The situation with respect to losses of nonalkaloidal nitrogen is somewhat clearer. Elementary chemical considerations (see equation, footnote 1) lead to the conclusion that the rate *in vivo* of Eggleton's proposed reaction and the extent of its occurrence may be influenced by the tissue concentrations as well as by the total potential supplies of primary amino nitrogen and of nitrate nitrogen (the latter a precursor of nitrite nitrogen in the cell). Furthermore, this reaction must compete with other reactions in the organism, such as assimilation and protein synthesis, which constitute the more nearly normal pathways of biochemical activity for nitrates and amino acids, respectively. From a purely empirical standpoint, it would thus appear that those environmental circumstances which permit appreciable accumulation of free amino acids, of nitrates, or of both, could also be expected to provide the best conditions for the occurrence of Eggleton's reaction scheme. That the tobacco shoots employed in Expts. 1, 2, and 3 did vary in their initial composition, according to the above expectations, is evident from the following figures. The mean nitrate nitrogen concentration in the whole shoot tissues at the beginning of each experiment was 2.18% for Expt. 1, 1.69 for Expt. 2, and 0.81 for Expt. 3. The corresponding figures for free primary amino nitrogen, exclusive of that of the amides (see below), were 0.33, 0.15, and 0.13%, respectively. The reasonably close relationship between the trends indicated by these values and the occurrence of nonalkaloidal nitrogen loss (Table I) is apparent.

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[3] Confidence limits for the quantities of nitrogen lost in Expt. 1 can be estimated. Variance for the means of duplicated assays by the Kjeldahl method for total nitrogen of leaf and stem samples was 0.72 and 1.33%^{2}, respectively. Variance for the mean dry weights of leaf and stem of eleven plants was, similarly, 0.0030 and 0.0020 g.^{2} From these figures may be calculated the standard error of measurement of total nitrogen for the whole shoot, ±4.97 mg. of nitrogen. The standard error of a difference between two means is thus ±7.0 mg. of nitrogen per shoot. Since the total nitrogen values for most of the samples in this experiment involve the means of more than two individual assays, the actual confidence limits would be somewhat narrower than the above figures indicate.

A possible clue to the identity of the environmental circumstances which led to extensive losses of *total* nitrogen is given by the inverse correlation between the magnitude of such loss and the time of year in which growth had occurred (without supplementary illumination) in the greenhouse prior to excision of the plants for experimental use (Table I). The greatest losses occurred from plants grown in November, December and January (Expt. 1); smaller losses from plants harvested in March (Expt. 2); and no losses at all from plants grown in April and May (Expt. 3). It is also worth noting the correlations with type of nitrogen loss. For instance, both alkaloidal and nonalkaloidal nitrogen in important amounts were lost from plants grown in the winter. An amount of nitrogen equivalent to the alkaloidal loss disappeared from the plants grown during the late winter and early spring; and neither type of loss occurred from plants grown during the later spring months.

If the environmental conditions which led to the differences in tissue concentrations of nitrate and of amino nitrogen that were described above and thence to nonalkaloidal nitrogen loss were those actually concerned with differences between the seasons (*e.g.*, light intensity, daylength, *etc.*) it should be possible to test the relationship by simple experiment. Thus, plants grown in late spring might reasonably be expected to exhibit nitrogen loss when supplied with excessive amounts of inorganic nitrate, of ammonia, or of both (3, 4, 5). It is of interest to note, therefore, that plants harvested in May, which showed no loss of intrinsic nitrogen when cultured on solutions of nicotine hydrochloride (Expt. 3 of (1)) or in distilled water at pH 3.4–3.6, could nevertheless be shown to lose large amounts of nitrogen when supplied with readily assimilable reduced nitrogen or with oxidized nitrogen at the same pH.

In Table II are contained the hitherto unpublished results of feeding excised tobacco (var. Rosenberg) shoots with 0.05 *M* solutions of the two salts. The total nitrogen loss resulting from uptake by stem and leaf of NH_4Cl, 22.16 mg. per shoot, was approximately half that resulting from the uptake of NH_4NO_3, 40.78 mg. These estimates of nitrogen loss are, as usual, based in part upon assay values for the designated solutes in the culture solutions, both original and residual.

Nicotine was supplied in these experiments as the monohydrochloride salt. Thus, the role of nicotine in the disappearance of nonalkaloidal nitrogen may well have been not unique but rather the source of such acidity as is required by Eggleton's scheme to liberate free nitrous acid by simple double decomposition reaction with nitrites within the cell. The quantitative evidence is in close agreement with this suggestion. Each molecule of the nicotine salt contained two atoms of nitrogen and one molecule of HCl. If the scheme applied, each molecule of escaped nitrogen must have contained one atom of nitrogen derived from nitrous acid. Obviously, then, one molecule of absorbed nicotine salt should have accounted for the loss of one molecule of gaseous nitrogen. In accordance with this reasoning the daily loss of nitrogen (smoothed data) in Expt. 1 exactly equaled the daily accumulation of nicotine nitrogen (Table III). Since, however, a little more than 20% of the total absorbed nicotine nitrogen was also lost, the total amount of HCl (calculated, 2.0; found by chloride assay 1.9 millimoles) absorbed by each shoot was somewhat in excess of the amount required to account for the nonalkaloidal nitrogen loss. This situation might perhaps have been expected. Obviously, if the total nicotine nitrogen accumulated is added to the total nicotine nitrogen lost, then the daily coincidence between this and the total nitrogen loss is again complete. It may be added that this quasi-stoichiometric agreement also renders less probable such explanations of the activity of nicotine hydrochloride in stimulating nitrogen loss as a catalytic or other more specific type of behavior.

The unique features of the results to be discussed below may very likely, therefore, be traced directly to a fortuitous combination of circumstances (relatively high tissue concentrations of nitrates and of free amino acids, and a source of buffered acidity) which favored the probability of nitrogen loss by the mechanism proposed by Eggleton. Whether these conditions ever occur simultaneously in intact plants, and whether important amounts of nitrogen are thus lost by crop plants under adverse circumstances of weather, manuring, *etc.*, is another problem and cannot be answered by the present data. It would seem, however, that the failure of other workers (7, 8) to obtain nitrogen losses during attempts to repeat the experiments of Pearsall and Billimoria (4, 5) may well have been due to failure to provide one or more of the above conditions.

Origin of the Lost Nonalkaloidal Nitrogen

Quantitative data on the changes within the various nitrogenous fractions of the shoot in Expt. 1 should reveal the sources of the large amounts of nonalkaloidal nitrogen that were lost. The general patterns of interchange among these fractions under conditions of culture for excised organs are well known from the work of Vickery and his associates (6). In the discussion that follows, the summation of values for nitrogen fractions in any one collection of shoot material has been subtracted from the smoothed value for total nitrogen in Expt. 1. To the extent that the error variance of one of the two subtraction terms is thus reduced, one consequence of such calculation is a somewhat more reliable estimate of so-called "undetermined" nitrogen. According to Vickery *et al.* (6), this fraction can be expected to contain the nitrogen of the peptides, purines and other minor constituents of green tobacco tissues. It is also necessary to recall, in connection with the following discussion, that earlier experiments (1) have shown absorbed nicotine nitrogen to have no appreciable effect upon the metabolism of other nitrogenous compounds in excised tobacco shoots during culture.

Eggleton's scheme requires that the total nonalkaloidal nitrogen loss be accounted for exactly by simultaneous losses of nitrate and primary amino nitrogen in equal amount. The total nitrogen loss in Expt. 1 at the end of 96 hr. was somewhat less than twice the change in nitrate nitrogen[4] (Table IV). On a daily basis, it will be noted that in all cases 50% of the total nonalkaloidal nitrogen loss (the latter equal in the smoothed data to the amount of nicotine nitrogen recovered) could have been obtained from nitrates that disappeared, and 50% from the most likely sources of amino nitrogen (the amino acids, in-

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[4] The standard error of measurement for nitrate nitrogen calculated in the same way as that for total nitrogen is ±1.42 mg. per shoot. The standard error for a difference between means is thus ±2.01 mg.

TABLE IV

Balance Sheet of Nonalkaloidal Nitrogen Changes in Excised Tobacco Shoots (var. Turkish) during Culture in the Light in Solutions of Nicotine Hydrochloride for Various Periods of Time

The data are recalculated from Expt. 1 of the original paper (1).

Hr. of culture	Fraction	Nitrogen losses		Available for metabolism 1–2	Nitrogen gains		Balance
		Amount 1	Required for N_2 formation[a] 2		Fraction	Amount	
0–24	Nitrate	6.73	4.64	2.09	Undetermined	2.27	
	Amino	6.91	4.64	2.27	Amide	0.73	
					Ammonia	0.63	
					Insoluble	0.73	
	Total	13.64	9.28	4.36		4.36	−9.28
24–48	Nitrate	5.09	5.18	−0.09	Amide	0.55	
	Undetermined	8.91	5.18	3.73	Amino	0.27	
	Ammonia	0.09		0.09	Insoluble	2.91	
	Total	14.09	10.36	3.73		3.73	−10.36
48–72	Nitrate	9.27	6.86	2.41	Amide	2.18	
	Undetermined	9.82	6.86	2.96	Amino	1.09	
					Ammonia	1.46	
					Insoluble	0.64	
	Total	19.09	13.72	5.37		5.37	−13.72
72–96	Nitrate	7.73	3.82	3.91	Amide	1.36	
	Insoluble	4.09	3.82	0.27	Amino	1.45	
	Ammonia	0.27		0.27	Undetermined	1.64	
	Total	12.09	7.64	4.45		4.45	−7.64

The values in this table are mg. of N per shoot.

[a] According to Eggleton's scheme.

soluble or protein fraction, and the undetermined fraction). Indeed, in the 24–48 and 72–96 hr. periods, the contributions of the two classes attain separately almost the theoretical maximum. The greatest loss of free amino nitrogen occurred during the first 24 hr. During the next 48 hr., the undetermined nitrogen fraction supplied the bulk of the reduced nitrogen for the reaction, and in the last 24 hr. the proteins became involved. It is perhaps possible that this order of utilization represents the order of conversion of the nitrogenous fractions to compounds containing free primary amino groups. Eggleton (2) found a lowered content of pepsin-HCl-soluble nitrogen in grass which had been manured with nitrites.

Since the total nitrogen lost in 96 hr. in these experiments was less than twice the loss of nitrate nitrogen, it becomes necessary to account for the discrepancy. One possibility, suggested by the work of Vickery (6), is that part of the nitrate having escaped reaction with amino groups at the nitrite stage of reduction may have been converted into ammonia, amides, or other forms of reduced nitrogen. This appears to have been the case. Ammonia nitrogen and the nitrogen of the amides (presumably, but not certainly, asparagine and glutamine) increased slowly but more or less regularly throughout the 96 hr. of culture and accounted quantitatively for the discrepancies just noted. Failure of the α-amino nitrogen of the amides, and the amide nitrogen of whatever glutamine was present, to react with HNO_2 is an apparent contradiction to the applicability of Eggleton's suggested mechanism; but the contradiction may be only apparent in view of our ignorance of the exact loci within plant cells of the systems which reduce nitrates and which synthesize amides.

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SUMMARY

It has been found that extensive nitrogen losses may occur from excised tobacco shoots when cultured on solutions of a nicotine salt. The nitrogen losses were of two types: one involved the loss of nicotine or of its metabolic products, while the other involved nonalkaloidal nitrogen. Occurrence of the latter type was correlated with time of year during which the plants were grown, with high tissue concentrations of nitrate and of amino nitrogen, and with a source of buffered acidity. Quantitative analytical data support but do not prove the possibility suggested by Eggleton that plants may lose nitrogen gas as a consequence of the reaction *in vivo* between nitrous acid and free primary amino groups.

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Minimal Protein Requirement of the Rat for Reproduction and Lactation[1]

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The minimal protein requirement of the rat for reproduction and lactation is unknown. A wheat–whole milk diet containing 14.4% protein (dry weight) supported many generations of rats, but if protein was added to the diet, the growth improved (1). A diet of natural foods, with a protein content of 15%, was apparently adequate for the production of 5 generations of rats without any obvious difficulties (2). However, an increase in the protein to 25 and 40% resulted in superior reproduction and lactation. It is customary to feed 18% crude casein diets during the reproductive period, and satisfactory results have been reported on purified diets with 24–30% protein (3, 4).

The growth of rats on diets ranging from 19.1 to 7.1% protein, supplied in the form of polished rice, red kidney beans, and casein, has been described (5). Protein was the limiting factor of the diets and methionine, the limiting amino acid. The minimal protein requirement for growth was apparently met by the diet containing 16.7% protein. Observations also made on these rats were: the time of sexual maturity in the female; the occurrence of estrus; sexual activity and fertility in the male and female; gestation and lactation.

The minimal protein requirement of the rat for reproduction and lactation is the object of this report.

EXPERIMENTAL

Diets and Supplements

The composition of the diets was given in the report on growth (5). The amounts of added vitamins were doubled during gestation and tripled during lactation. The addition to the diets of niacin, Ca pantothenate, inositol, choline, PABA, folic acid, and vitamin K altered neither growth nor reproduction in a second series of rats on the various protein levels. Since manifestations of vitamin E deficiency were detected in the first series, α-tocopherol was given to all other rats on the rice-beans-casein diets, 1 mg. per week to males during the period of growth and 10 mg. to females at the beginning of each gestation.

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Procedure

During the period of growth, the occurrence of estrus was determined by daily examinations of the vaginal contents (6). The age and body weight at the time of first estrus and the length of intervals between periods of estrus were also noted.

The rats were bred at about 90 days of age, or after they had attained a body weight of 170 g. During estrus, the females were paired with males on the same diet, except in the case of low-protein diets, when males and females were bred with normal breeding rats of proved fertility. Impregnation was detected on the following morning by an examination of the vaginal contents for the presence of the vaginal plug and the occurrence of spermatozoa. During the 13th and 14th days of gestation, the blood clot in the vagina, which is an infallible sign of implantation of ova on the uterine wall, was looked for.

The females were weighed at the beginning of pregnancy, on the 14th day, and daily after the 19th day. Normally, parturition occurred on the 22nd day. Litters were examined for size, sex ratio, number of living, and birth weight of young. In cases of abnormal parturition, the females were killed. All rats which died or were killed were examined for dead fetuses and for the number of resorption sites in the uterus.

Litters were not reduced in size during the period of lactation. Observations were made on the number of young surviving the period and on the increase in body weight of female and young, which were weighed twice a week, on the 17th day, at which time the young began to eat the maternal diet, and on the 21st day, when the young were separated. The females were permitted a recuperative period of two weeks between pregnancies.

If three different females which were bred with a male failed to present the implantation sign after insemination, the male was killed and the contents of the vas deferens in Locke's Ringer solution were examined for mature motile spermatozoa.

Food consumption was measured during the first 21 days of gestation and 17 days of lactation.

DISCUSSION

Since standards need to be defined, reproduction and lactation will be considered normal in this report if they are similar to those that have been observed in four generations of rats on an adequate diet under the given laboratory conditions (7).

Estrus. Estrus data of first and second generation rats are presented in Table I.

TABLE I

Estrus in Rats on the Rice-Beans-Casein Diets

	Per cent protein							
	24.8 (7)	25.2	19.1	16.7	14.3	11.9	9.5	7.1
Age in days at time of first estrus								
First generation								
Number of determinations	47	6	6	12	12	12	12	11
Mean days	37.9	35.7	41.5	38.5	43 2	49.2	65.5	105.1
Probable error	±0.5			±0.5	±1.1	±1.2	±2.0	±3 2
Second generation								
Number of determinations		22		6	9	10		
Mean days		40.6		42 2	47.3	53.1		
Probable error		±0.4						
Body weight in g. at time of first estrus								
First generation								
Number of determinations	47	6	6	12	12	12	12	11
Mean g.	90.7	97 5	115 8	102.9	110.6	119.0	124.5	129 0
Probable error	±1.2			±1.5	±2.9	±2.6	±3 2	±2 9
Second generation								
Number of determinations		22		6	9	10		
Mean g.		99.9		108.3	102 9	92 3		
Probable error		±1 9						
Length in days of estrus cycle								
First generation								
Number of determinations	492	57	53	111	99	84	38	98[a]
Mean days	4.8	5 8	5 3	5 6	5 7	5 9	8 2	7.8
Probable error	±0 03	±0 20	±0.18	±0.17	±0 17	±0.16	±0 52	±0 27
Second generation								
Number of determinations		198		53	86	75		
Mean days		5.4		4.9	5.0	5.2		
Probable error		±0 10		±0.11	±0.11	±0.09		

[a] After 84 days of age.

Age in Days at the Time of First Estrus. In the animals on diets containing 16.7%, or more, of protein, the first estrus occurred at about 36-42 days of age. With diminishing dietary protein, there was a delay in the onset of maturity, which was not very great in first generation rats on the 14.3 and 11.9% protein diets. The delay was significant in females of the second generation on these diets, and in rats on the lower protein levels. It was associated with retardation in the rate of growth (5) as reported by Evans and Bishop (8). The mean body weight at the time of first estrus tended to be more constant than the mean age.

Length of Intervals between Periods of Estrus. Estrus occurred regularly at intervals of 4–6 days in first and second generation rats receiving 11.9%, or more, of protein. Frequently, the first interval between estrus periods was longer than 6 days. Upon lower protein intake, the intervals between periods were longer.

Although second generation rats on diets containing 14.3 and 11.9% protein were retarded in growth (5), the length of intervals between estrus was within normal limits. Usually, stunting due to nutritional inadequacy results in fewer estrus periods (8), but it appears that, on restricted protein diets, retardation in growth may occur without changes in the length of the interval between them.

Fertility. Fertility was expressed as the per cent of rats bred that sired or cast litters, regardless of litter size or number of living young; furthermore, a female that lost 15 g., or more, in body weight during the 22nd day of gestation was assumed to have cast young.

Males. In Table II, a high percentage of males on diets containing 11.9%, or more, of protein sired litters. On the 9.5% protein diet the fertility decreased to 50%.

Sterility in males was associated with failed insemination and vitamin E deficiency. Two males failed to impregnate females, though each had been paired with at least 6 females in which fertility was proved. The males were killed and presented large, firm testes and many motile spermatozoa. This type of sterility, reported by Beach (9), had no relation to dietary protein. In 2 males on the 11.9% and 3 males on the 9.5% protein, there were observed the small watery testes and atypical spermatozoa, characteristic of vitamin E deficiency (10, 11). Such sterility was prevented in other males on the rice-beans-casein diets, by the administration of 1 mg. α-tocopherol weekly during the period of growth.

Females. Living young were produced by females on all diets containing 11.9%, or more, of protein. On the lower protein levels, most of the young were dead at the time that the litters were found, or resorption gestations occurred.

Sterility in females was associated with failed impregnation or implantation, or with vitamin E deprivation. Two females failed to become impregnated, though each was paired with at least 6 males of proved fertility. As in the males, this type of sterility (9) was not related to protein concentration of the diet.

There were 9 females in which insemination never resulted in implantation, though each one had been impregnated at least 3 times by males of proved fertility. This high incidence of failed implantation increased during the second and third reproductive periods and was observed not only in females on the rice-beans-casein diets but also in normal controls. In 5 of the older females there was gross evidence of

TABLE II

Fertility and Sexual Activity of Rats on the Rice-Beans-Casein Diets

	Per cent protein							
	24.8 (7)	25.2	19.1	16.7	14.3	11.9	9.5	7.1
Males								
Fertility								
Number bred	29	6	6	12	12	12	8	0
Number siring litters	23	6	6	11	12	10	4	
Per cent	79	100	100	92	100	83	50	
Cause of sterility								
Number with								
Failed impregnation	3	0	0	1	0	0	1	
Vitamin E deficiency	3	0	0	0	0	2	3	
Sexual activity of fertile rats								
Number of pairings with females	34	7	10	23	27	17	4	
Number of impregnations	26	6	6	14	16	13	4	
Per cent	76	86	60	61	59	76	100	
Females								
Fertility								
Number bred	63	6	6	12	12	12	12	8
Number with litters	62	6	5	10	10	10	10	0
Per cent	98	100	83	83	83	83	83	0
Cause of sterility								
Number with								
Failed impregnation	1	0	1	0	1	0	0	0
Failed implantation	0	0	0	2	1	2	2	2
Vitamin E deficiency	0	0	0	0	0	0	0	6
Litters of dead young	0	0	1	0	0	0	8	0
Sexual activity of fertile rats								
Number of pairings with males	75	8	5	18	21	11	13	
Number of impregnations	65	8	5	10	12	7	12	
Per cent	87	100	100	56	57	64	92	

oviduct infection. All breeding males were killed. They presented normal appearing testes and motile spermatozoa. This type of sterility in females, reported on vitamin E-low diets (12, 13), was presumably related to faulty technique in sampling the vaginal contents. It did not appear in other groups of rats.

Resorption gestation, typical of vitamin E deficiency, occurred in the rats on the 7.1% protein diet. In order to assure adequate supplies of vitamin E, 10 mg. α-tocopherol was given to all rats on the rice-beans-casein diets at the beginning of each gestation.

Sexual Activity. Data on the sexual activity of male and female rats until the production of the first litter are presented in Table II. The records of sterile rats were omitted. There was great variation among

TABLE III

Reproduction in Rats on Rice-Beans-Casein Diets

	Per cent protein							
	24 8 (7)	25.2	19.1	16.7	14.3	11.9	9 5	7.1
Number of gestations	62	34	11	20	18	15	10	6
Number of litters with living young	62	28	8	18	17	11	2	0
Gestation Index[a]	100	83	73	90	94	73	20	0
Number of litters alive on 4th day	57	25	8	14	14	8	0	
Viability Index[b]	92	89	100	78	82	73	0	
Overall Reproduction Index[c]	92	73	73	70	78	53	0	0
Litters with living young								
Mean number per liter	8.7	8.8	9.2	7.6	7.2	5.4	2.5	
Per cent living	99	96	89	85	80	66	80	
Mean birth weight in g.								
Males	5.6	5.8	5.4	5.4	5.4	5.4	5.0	
Females	5.3	5.6	5.3	5.3	4.8	5.2	4.7	
Body weight in g.								
Initial, of female	196	206	219	215	216	200	201	
Gain, female + young	91	85	96	84	79	69	39	
Gain, female only	36	27	34	32	30	31	19	

TABLE III—*Continued*

Addition of 10 mg. α-tocopherol per gestation

	Per cent protein							
	24.8 (7)	25.2	19.1	16.7	14.3	11.9	9.5	7.1
Number of gestations				5	17	10	3	
Number of litters with living young				5	16	10	3	
Gestation index[a]				100	94	100	100	
Number of litters alive on 4th day				5	16	10	2	
Viability index[b]				100	100	100	67	
Overall Reproduction index[c]				100	94	100	67	
Litters with living young								
Mean number per litter				8.2	7.2	5.2	6.5	
Per cent living young				100	99	98	92	
Mean birth weight in g.								
Males				6.0	5.7	5.8	5.4	
Females				5.9	5.4	5.9	5.0	
Body weight in g.								
Initial, of female				192	188	177	172	
Gain, female + young				89	77	77	51	
Gain, female only				36	24	36	−1	

Indices of Mirone, Panzarella and Cerecedo (14):

[a] $$\text{Gestation Index} = \frac{\text{Number of litters born alive}}{\text{Number of gestations}} \times 100.$$

[b] $$\text{Viability Index} = \frac{\text{Number of litters alive on 4th day}}{\text{Number of litters born alive}} \times 100.$$

[c] $$\text{Overall Reproduction Index} = \frac{\text{Number of litters alive on 4th day}}{\text{Number of gestations}} \times 100.$$

individuals, but it is evident that the concentration of protein in the diet did not influence the percentage impregnation of either male or female rats when males were bred with females in estrus.

Gestation. Although there was great variation among rats, there was no apparent difference in gestation between first, second, and third litters, or between first, second, and third generation rats. The data were, therefore, combined. Since vitamin E deficiency was detected in the first series of rats, females were divided into 2 groups; those that obtained their supply of vitamin E from the rice-beans-casein diets, and those that received, in addition, 10 mg. α-tocopherol at the beginning of gestation. The indices suggested by Mirone, Panzarella and Cerecedo (14) for reproduction and lactation in the mouse were used.

The reproductive failure of rats on the rice-beans-casein diets, without the addition of α-tocopherol, is evident in Table III. On the lower protein diets, there was a progressive decrease in litter size, in the number of living young and in birth weight.

As will be seen by the effect on the indices of gestation, viability, and overall reproduction, of the administration of α-tocopherol at the beginning of gestation, the failure in reproduction was not associated with protein intake. With adequate vitamin E, on all protein levels, except that of 9.5%, the number of living young and the birth weight were within normal limits. The size of the litter, however, was reduced on restricted protein diets. The decrease in litter size accounted for the smaller gain in total body weight of females. There was approximately the same net gain in body weight during the gestation period in all females, except those on the 9.5% protein diet.

Parturition occurred on the 22nd day in rats on all diets, except in a few instances, when females died on the 23rd or 24th day of gestation. Severe uterine hemorrhage was observed in some rats, lung infection in others. Two females on the 14.3%, and 4 females on the 11.9%, protein diets presented the liver necrosis characteristic of methionine deficiency.

It appears that, under the laboratory conditions given, the rice-beans-casein diet containing 16.7% protein is the minimal protein diet that supports reproduction equal to that of control rats on an adequate diet.

Lactation. Since the administration of α-tocopherol at the beginning of gestation apparently did not influence lactation of rats on the rice-beans-casein diets, the lactation data were combined. Litters in which the young did not survive the period of lactation were not included. The data on the 9.5% protein diet were given in order to show that lactation is possible at this low protein concentration.

The lactation of females on the diets containing 16.7 and 19.1% protein was similar to that of the controls, except in the number of

TABLE IV

Lactation in Rats on Rice-Beans-Casein Diets

	Per cent protein						
	24.8 (7)	25.2	19.1	16 7	14.3	11.9	9.5
Number of litters alive on 4th day	57	25	8	19	26	18	2
Number of litters weaned	54	25	8	19	25	17	1
Lactation Index[a]	95	100	100	100	96	94	50
Young							
Number surviving per litter	8.0	8.0	6.6	6.6	5.9	3.8	4.0
Per cent	93	92	86	89	82	81	100
Mean body weight in g. at at 21 days							
Males	35.9	31.2	35.8	33.7	27.1	29.6	10.0
Females	34.2	31.0	35.8	33.9	29.1	27.3	0
Body weight in g.							
Initial, female + young	277	285	297	283	264	251	195
Initial, female only	230	234	253	243	225	223	163
Gain, female + young	156	117	112	106	68	34	−2
Gain, female only	−10	−17	−16	−11	−16	−18	−1

[a] Lactation Index (14) = $\frac{\text{No. of litters weaned}}{\text{No. of litters alive on 4th day}} \times 100.$

surviving young. The effect of protein restriction, evident on the 14.3% protein concentration, was a reduction in the number of surviving young and in the rate of growth of the survivors. The body weight of the young at 21 days was influenced by the number of sucklings per litter. When all of the young survived, they tended to be smaller in size and to appear undernourished. When some of the young died at the beginning of lactation, which frequently occurred, the surviving young were larger and appeared to be normal. Females on all diets lost weight during the period of lactation. The gain in total body weight of female and young was greater in controls than in rats on the rice-beans-casein diets, and was related to the number of survivors and their rate of growth.

It is interesting that the second and third generation rats on 14.3 and 11.9% protein diets were normal in appearance, yet small in stature. The birth weight of these animals was the same as that of controls on an adequate diet, but the rate of growth was significantly retarded during the periods of lactation and of rapid growth (5).

The findings are in accord with the observations of Mueller and Cox (15). They analyzed the milk of rats on low-protein intake and concluded that there was no significant change in per cent protein in the milk, but the volume of milk was greatly reduced. This necessarily limited the growth and weight of the young.

Most of the young which did not survive the period of lactation died during the first 5 days: some apeared to be malnourished; a few presented gross necrotic liver lesions; neither symptoms nor gross lesions of muscle degeneration, such as are associated with vitamin E deprivation (16, 17, 18) during lactation, were observed.

Food Consumption. Although the minimal protein requirement for growth, reproduction, and lactation was apparently met by the same

TABLE V

Food Consumption of Rats during Periods of Gestation and Lactation on Rice-Beans-Casein Diets

	Per cent protein					
	24.8 (7)	19.1	16.7	14.3	11.0	9.5
Daily food consumption						
Period before gestation, 7 days						
Number of determinations	12	6	12	12	12	12
Mean g. food	8.5	11.2	10.3	10.1	10.2	9.6
Mean number calories	36	38	35	34	35	33
Mean g. protein	2.1	2.1	1.7	1.4	1.2	0.9
Gestation, 21 days						
Number of determinations	28	8	18	17	11	2
Mean g. food	13.0	15.0	14.0	13.9	12.8	10.8
Mean number calories	50	51	47	47	44	37
Mean g. protein	3.2	2.9	2.3	2.0	1.5	1.0
Lactation, 17 days						
Number of determinations	19	8	14	10	8	
Mean g. food	22.0	21.9	20.7	17.9	14.8	
Mean number calories	90	74	70	61	50	
Mean g. protein	5.5	4.2	3.4	2.6	1.8	

diet, there was an increase in the daily food consumption of rats during the periods of gestation and lactation. During the week before gestation, the daily caloric intake of females on the rice-beans-casein diets containing 11.9%, and more, protein was approximately the same as that of the controls, about 35 Cal.

During gestation, the caloric intake of the controls and of the rats on the 19.1% protein increased to 50 Cal., but the amount was less in rats on the lower protein diets. Food consumption increased on all protein levels, from the day of impregnation, but there was no further increase during the last week, when the body weight was increasing greatly. Very little food was eaten on the day of parturition (22nd day). During lactation the caloric intake of the control rats increased to 90 Cal. per day, while that of the rats on the rice-bean-casein diets was less than 75.

The lowered food consumption on the rice-beans-casein diets may be accounted for by possible differences between them and the control diet in palatability, digestibility, and availability of nutrients. It may have been impossible for the rats to eat sufficient food to obtain the needed calories. Perhaps the substitution of fat for carbohydrate in isodynamic proportions might enable the rats to increase their caloric intake, with resulting improvement in lactation (19).

The 16.7% protein diet, which was apparently the minimal protein diet for reproduction and lactation, contained 7.8 mg. T.N./Cal. The daily protein intake of rats on this diet during gestation was 2.3 g. and during lactation, 3.4 g.

SUMMARY

Rats were observed during the periods of reproduction and lactation on diets ranging from 19.1 to 7.1% protein, supplied by a certain mixture of polished rice, red kidney beans, and casein. Protein was apparently the limiting factor of the diets, and methionine, the limiting amino acid.

1. The rice-beans-casein diet did not contain adequate vitamin E for reproduction.
2. With the addition of α-tocopherol, the minimal protein concentration which supports reproduction and lactation (excepting the number of surviving young) in several generations of rats, such as that obtained on an adequate diet, was 16.7%. This was also the minimal protein diet for growth. It contained 7.8 mg. T.N./Cal.

3. There was an increase in daily food consumption during gestation of 33% above the amount consumed by the female before reproduction, and during lactation, of 100%. The food intake during lactation was not as great as that of the control rats, which increased 150%, and resulted in a decrease in the number of surviving young.

4. The concentration of protein in the diet apparently had little effect upon the frequency of estrus periods, sexual activity, fertility, or the body weight of young at birth.

5. Restricted dietary protein was associated with a delay in the occurrence of the first period of estrus, a decrease in litter size, and a reduction in the rate of growth of young during, and in the number of young surviving, the period of lactation.

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The Reaction of Mustard Gas with Skin Proteins [1]

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INTRODUCTION

The reaction of mustard gas with proteins was first clearly demonstrated by the work of Berenblum and Wormall (1). During World War II ample confirmation of this finding was forthcoming from both British and American laboratories. Much of this work still remains unpublished, but some of it may be found in Refs. (2–9). In view of the postwar interest in both the sulfur and nitrogen mustards from the standpoint of production of mutations (9,10) and the treatment of leukemia (10–14), the wartime vesicant gas studies take on added interest and prompt our publication of these results.

One of the most fruitful methods in the study of the mechanism of the vesicant action of mustard gas has been the use of gas prepared from radioactive sulfur. Among the earliest observations of Henriques (15) employing radioactive mustard (H*) was that a definite percentage of the radioactive sulfur of the vesicant which penetrated the skin always remained in the skin tissue while the largest portion was carried away by the body fluids. The radioactive material which remained behind in the skin appeared to be a reaction product of H* with some constituent of the skin, since it could not be extracted from the excised minced tissue by use of organic solvents. In view of the fact that this "fixed mustard" might be concerned in the reactions leading to vesication, it appeared worthwhile to attempt to learn more about its nature. The experiments to be described here were therefore undertaken with this end in view.

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[1] This work was done under Contract No. OEMsr-86 between the Office of Scientific Research and Development and the President and Fellows of Harvard University during the period February 1 to May 1, 1942.

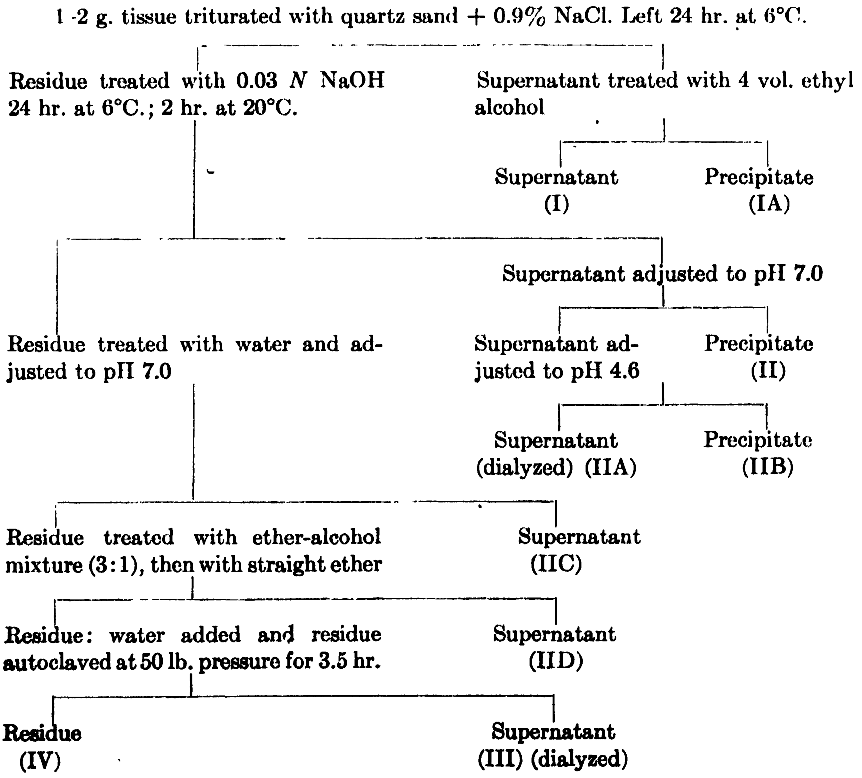
Procedure

The skin samples fractionated in this study were obtained by the following procedure. Six to ten mg. of H*/5 cm.[2] were applied to the skin of a young pig on the side of the abdomen or the outer aspect of the thigh. The area so treated was covered with a taped-on watch glass for 6 hr. At the end of this period, the watch glass was removed, the H* remaining on the skin surface wiped off with cotton pledgets soaked in petroleum ether and 24 hr. allowed to elapse. At the end of this time the pig was anesthetized, the burned areas frozen with CO_2 snow and cut out with a sharp cork borer. The skin samples employed were trimmed free from muscle and fat, and were mainly composed of the epidermis and corium.

Application of the usual procedures to the mincing of pig skin tissue were soon found to be inadequate. The procedure finally adopted was to cut the frozen samples into as fine slivers as possible with a razor blade; these were then mixed thoroughly and aliquots taken for fractionation.

Exhaustive acetone treatment of aliquots at this point indicated that only negligible amounts of radioactive sulfur could be extracted. This step was, therefore, abandoned in obtaining the main fractionation data, since it was desired to avoid any process that might lead to the denaturation of the water-soluble skin proteins.

Flow Sheet of Skin Fractionation Procedure


1-2 g. tissue triturated with quartz sand + 0.9% NaCl. Left 24 hr. at 6°C.
Residue treated with 0.03 N NaOH 24 hr. at 6°C.; 2 hr. at 20°C.
Supernatant treated with 4 vol. ethyl alcohol
Supernatant (I)
Precipitate (IA)
Supernatant adjusted to pH 7.0
Residue treated with water and adjusted to pH 7.0
Supernatant adjusted to pH 4.6
Precipitate (II)
Supernatant (dialyzed) (IIA)
Precipitate (IIB)
Residue treated with ether-alcohol mixture (3:1), then with straight ether
Supernatant (IIC)
Residue: water added and residue autoclaved at 50 lb. pressure for 3.5 hr.
Supernatant (IID)
Residue (IV)
Supernatant (III) (dialyzed)

The fractionation procedure employed is given on the accompanying flow sheet. The skin samples were first thoroughly ground in a mortar with quartz sand and 0.9% NaCl for about 0.5 hr. in order to extract all water-soluble materials and to reduce the preparation to a fine suspension. The water-soluble proteins were then precipitated by addition of ethyl alcohol (Fraction IA). Next, the water-insoluble residue was treated with dilute alkali to extract material other than collagen and elastin. In this fraction should be found the nucleoproteins. The procedure from here on followed largely that outlined by Lowry *et al.* (16) for the determination of collagen and elastin. We have, however, at two stages of the fractionation (IIA and III) carried out dialysis in order to ascertain whether or not the radioactive sulfur at these stages was actually bound to an impermeable molecule such as protein. Dialysis was carried out for 24 hr. periods at 6°C. on a rocker type dialyzer, using cellophane membranes.

Each of the fractions so obtained was subjected to an analysis for its dry weight. total phosphorus, and radioactive sulfur content. Dry weights were determined by drying at 102–105°C. for 24 hr. A correction for NaCl in Fractions IA and IIA was made. Total phosphorus was determined by the method of Fiske and SubbaRow (17), while the radioactive sulfur was determined as described by Henriques *et al.* (18). Phosphorus determinations were made in order to see whether there was any correlation between "fixed mustard" and phosphate ester distribution in the various fractions. All values were determined in duplicate.

RESULTS

The results of the analysis of the fractions indicated in the preceding flow sheet are given in Table I.

As can be seen from the data recorded in this table, only about 17% of the total skin material is soluble in 0.9% NaCl, 10% can be brought into solution by treatment with 0.03 *N* NaOH, 8% is soluble in alcohol-ether mixture, and the remaining 65% is to be classified as collagen and elastin. This distribution of material is almost identical with that found for a normal piece of pig skin from the same animal that was carried through the same fractionation procedure. The treatment with H* has, therefore, not markedly affected the solubility characteristics of the skin constituents.

The total amount of fixed mustard in this sample of tissue was 558 γ. This corresponds to a little over 0.1% of the total dry weight of the sample. This relatively large amount reflects the fact that the burn was a severe one. The data indicate that very little H* is attached to those skin proteins soluble in 0.9% NaCl (Fraction IA). Nearly all of the radioactive sulfur appears in the two fractions, IIA and III. Most surprising is the fact that the largest percentage of the radioactive sulfur in these fractions is dialyzable. This must mean that the fractionation

TABLE I

The Percentage Distribution of Dry Weight, Radioactive Sulfur (S) and P in Various Fractions of Pig Skin*

Fraction	Dry weight	Total phosphorus	Radioactive sulfur
I	14.2	28.4	10.5
IA	3.2	42.5	3.6
II	1.9	0.0	1.6
IIA	4.8	4.5	46.7 (dialyzate)[a] 6.0 (impermeate)[a]
IIB	0.5	0.0	0.4
IIC	2.8	0.0	1.3
IID	7.7	11.6	0.4
III	53.8	13.5	12.8 (dialyzate) 18.0 (impermeate)
IV	11.2	0.0	1.4
Total amount in all fractions	477.3 mg.	785 γ	558.3 γ[b]

[a] Dialyzate is defined as material in solution that can pass through a semipermeable membrane. Impermeate is defined as the material that cannot pass through a semipermeable membrane.

[b] This value is expressed as the total amount of H* that has become fixed in this tissue sample. It is a calculated value obtained by using the radioactive sulfur content of the tissue and the known radioactive sulfur of the applied mustard.

procedure has split off the fixed mustard from a skin constituent insoluble in 0.9% NaCl. In the case of Fraction IIA, nearly 50% of the total fixed mustard has been split off by the mild treatment with cold 0.03 *N* NaOH. The split product was identified as largely thiodiglycol by means of partition coefficient studies. A very labile linkage must, therefore, exist between this portion of the fixed mustard and the skin constituent to which it is attached. It is, of course, impossible to say from which fraction this radioactive thiodiglycol has been split. Indeed, all of it may have come from fractions which themselves remain insoluble as the result of the alkali treatment.

It is obvious that mustard must react with many different constituents of skin tissue, for there is at least a small amount of radioactive sulfur in every fraction. There is also evidence for differences in the type of linkages involved in the fixation of mustard in the fact that only part of the total radioactive sulfur is split off by any one procedure.

No obvious correlation between the distribution of total phosphorus and radioactive sulfur in the various fractions can be discerned. About 71% of the total phosphorus appears in Fractions I and IA, while these same fractions contain only 14% of the total radioactive sulfur. On a molar basis, however, there is, in the remaining fractions, still twice as much phosphorus as radioactive sulfur. No conclusion can, therefore, be drawn as to the possible role of phosphorus compounds in the fixation of mustard. Analysis of the phosphorus content of the various fractions of untreated skin showed that Fraction IA contained twice as much phosphorus as this same fraction from H*-treated skin. Whether this lower content of water-soluble phosphorus in treated skin is significant we hesitate to conclude in view of the single observation.

In view of the above findings, it seemed worthwhile to investigate further the percentage of fixed mustard which can be liberated by treatment with dilute alkali and by autoclaving.

The effect of successive extractions of skin with 0.03 *N* NaOH was first investigated. An aliquot of H*-treated skin was first extracted with cold acetone to remove lipides and any thiodiglycol that might be present. It was then ground with quartz sand in 0.03 *N* NaOH and allowed to stand for 24 hr. at 6°C. The process was repeated twice, and each of the 3 resulting extracts dialyzed for 24 hr. at 6°C. The various fractions were then analyzed for radioactive sulfur.

The results are given in Table II.

TABLE II

Effect of Successive Extractions of H-treated Pig Skin with 0.03 N NaOH*

	Radioactive S extracted γ	Percentage of total extracted
1st NaOH extract, dialyzate	51.0	55.0
1st NaOH extract, impermeate[a]	37.0	39.0
2nd NaOH extract, dialyzate	2.0	2.0
2nd NaOH extract, impermeate	lost	lost
3rd NaOH extract, dialyzate	1.5	2.0
3rd NaOH extract, impermeate	2.0	2.0
Total extracted	93.5γ	

Unextracted = 105.0γ.
Total fixed H = 204.5γ/g. of tissue

[a] The term impermeate is used to describe the material which does not pass through the cellophane dialysis sac.

A negligible amount of radioactive material appears in the acetone extract. This finding, coupled with the results on the alcohol-ether fraction (IID) of Table I, indicates that fixed mustard is definitely not associated with lipoid material in the pig skin.

It is obvious that the first extraction with 0.03 *N* NaOH removed nearly all (94%) of the radioactive material that can be extracted by this procedure. The total amount of radioactive sulfur obtained in all 3 extracts constitutes 47% of the total fixed sulfur present in this skin sample. This is in fair agreement with the results of the experiment which are given in Table I, where 52.7% of the total was obtained in the 0.03 *N* NaOH fraction. However, somewhat less of the extracted material is in a dialyzable form in this experiment than in the previous one.

It would appear, therefore, that not much more that 50% of the total fixed mustard of skin can be brought into solution by cold 0.03 *N* NaOH. More than half of this is, however, in a dialyzable form.

The amount of radioactive sulfur extractable from pig skin by autoclaving was next determined. An aliquot of skin was extracted with acetone and triturated with quartz sand and distilled water. The suspension was then autoclaved at 50 lb. steam pressure for 4 hr. The preparation was next centrifuged, the residue washed once with water, and the supernatant and washings pooled and dialyzed 24 hr. at 6°C.

Table III gives the results of the analyses of the various fractions for radioactive sulfur.

TABLE III

The Effect of Autoclaving on H-Treated Pig Skin*

	Percentage of total radioactive S
Autoclaved supernatant, dialyzate	69.0
Autoclaved supernatant, impermeate	24.0
Autoclaved residue	7.0

Total fixed H = 354.7γ/g. of wet tissue.
Acetone extraction yielded 7.0γ of H/g. of wet tissue.

It will be seen that autoclaving at neutral pH brings into solution 93% of the total "fixed mustard" in the skin, 69% being in a dialyzable form. The fractionation procedure previously employed (Table I) brought 98.6% into solution, 59.5% being in a dialyzable form. It would appear, therefore, that nearly all of the "fixed mustard" in skin may be brought into solution, with two-thirds of it in a dialyzable form, by the treatments described here. This dialyzable material is presumably nearly all thiodiglycol.

Finally, it was deemed worthwhile to make one more attempt to learn what role phosphate compounds might play in the fixation of mustard in skin tissue. If mustard becomes attached to skin constituents through a phosphate ester type of linkage, it might be possible to split it off by the action of the enzyme phosphatase. This possibility was, therefore, tested by treating H*-burned skin with a concentrated preparation of an alkaline phosphatase which was known to act upon adenyl pyrophosphate, phosphopyruvate, and which possessed phosphodiesterase activity when tested on yeast nucleic acid. The enzyme was prepared from calf intestine and kindly supplied to us by Dr. Gerhard Schmidt.

An aliquot of H*-treated skin was triturated with quartz sand and 0.9% NaCl. The supernatant was then treated with alkaline phosphatase in $M/20$ sodium veronal buffer, pH 8.9, for 24 hr. at 6°C. Tests with β-glycero-phosphate as substrate showed the enzyme to be strongly active at 6°C. The mixture was then dialyzed at 6°C. for 24 hr. The 0.9% NaCl-insoluble residue was similarly treated with enzyme. After 24 hr., the preparation was centrifuged, the residue washed and the supernatant and washings also dialyzed at 6°C. for 24 hr. Controls, to which only veronal buffer was added, were run on all fractions. Analysis of the various fractions was made for radioactive sulfur and total phosphorus.

The results of the radioactive sulfur analyses are given in Table IV.

TABLE IV

The Action of Phosphatase in N/20 Sodium Veronal Buffer, pH 8.9, on Pig Skin

Fraction[a]	Radioactive S content Enzyme-treated *per cent*	 Controls (buffer only) *per cent*
NaCl extract, dialyzate	3	1
NaCl extract, impermeate	2	3
Residue supernatant, dialyzate	42	50
Residue supernatant, impermeate	1	2
Insoluble residue	52	44
Total amount in all fractions	433γ[b]	377.5γ[b]

[a] See text for description of fractions.

[b] Expressed as H fixed per gm. wet tissue.

It is obvious that the interpretation of the action of phosphatase upon the fixed mustard of the skin is complicated by the surprising action of the weakly alkaline buffer itself. There is brought into solution by the action of the veronal buffer alone, 52% of the total radioactive sulfur material in this skin sample. Since treatment with 0.03 *N* NaOH was found previously to dissolve 51% of the material containing radioactive sulfur, it is thus no more effective than the more weakly alkaline buffer. Moreover, nearly all of the material brought into solution by the action of the buffer is dialyzable. It would appear, therefore, that at least 50% of the fixed mustard in the skin tissue is held by an extremely labile linkage. In view of the fact that the presence of phosphatase does not increase this fraction, it may be concluded that the remaining bound mustard is at least not susceptible to the action of phosphatase. No conclusion can of course be drawn concerning the action of phosphatase on that portion which is split off.

Phosphorus analyses indicated that the only significant difference in the distribution of phosphorus in the various fractions of the enzyme treated and control portions occurred in the material extracted with 0.9% NaCl. There is some increase in the dialyzable phosphorus of this fraction after treatment with phosphatase suggesting that the enzyme has split certain phosphate linkages.

As shown in Table IV, there is also an increase in the amount of dialyzable radioactive material. The total amount of radioactive material in this fraction is, however, small and the significance of the results in relation to the action of the enzyme is, therefore, debatable. It may be pointed out, however, that there should be extracted in this fraction those enzymes and coenzymes concerned in the phosphorylation mechanisms of the skin which have been shown to be inactivated by treatment with mustard *in vitro.*

DISCUSSION

The results given here indicate that the largest portion of the so-called "fixed mustard" in pig skin is attached to constituents insoluble in weak salt solutions. It seems likely, though it is not proved, that the structural proteins of the skin are chiefly concerned in the reaction. The exact nature of the linkages of mustard with these skin proteins is, however, not known. In view of the results obtained above, at least two different types of linkage would seem to be involved. One is a linkage which is readily split by treatment with dilute alkali and heat to yield a dialyzable sulfur residue which appears to be thiodiglycol. Some 60% of the total fixed mustard in skin is accounted for by this type of linkage. The other linkage is resistant to treatment with alkali and heat, and the protein to which the mustard is attached is brought into solution by such treatment.

It would seem reasonable that the alkali-labile linkage is one involving the carboxyl groups of the proteins. Since the preliminary reports of this work were made, there have appeared many observations to indicate that carboxyl groups of proteins will react with mustard gas to form an ester type of linkage. For example, Herriott, Anson and Northrop (6) have shown that the sulfur content of many proteins may be increased by treatment with mustard and that this extra sulfur is split off by treatment with alkali. Titration curves of such mustard-treated proteins also indicate that carboxyl groups are involved in the reactions. du Vigneaud and his coworkers (7,8) have confirmed the alkali lability of a portion of the mustard that becomes attached to various proteins. Davis and Ross (3) have shown that as many as 30 molecules of mustard may be added to each molecule of hemoglobin. Titration curves of the treated hemoglobin showed that 50–70% of the protein groups that had reacted were titratable in the pH range 2.0–5.5 and were thus presumably carboxyl. The predominant reactive group in isolated individual proteins would thus appear to be the carboxyl group. The fact that the alkali-labile linkage constitutes the largest percentage of those found in skin tissues is, therefore, in keeping with the interpretation that it also involves carboxyl groups.

The nature of the linkage or linkages resistant to treatment with alkali and heat in our experiments is not clear. Davis and Ross (3) have presented evidence to indicate that the imidazole group of histidine residues in the hemoglobin molecule reacts readily with H. Other linkages that may be concerned involve the hydroxyl group of tyrosine residues and the sulfhydryl group of cysteine. Herriott *et al.* (6) have shown that pepsin treated with H gives less color with the Folin phenol reagent but interpret this as a secondary effect caused by H reacting with protein carboxyl groups. Banks *et al.* (4) have shown that a marked reduction in the detectable SH groups of proteins occurs after their treatment with mustard.

How many of these linkages are involved in the fixation of mustard by the skin proteins cannot be estimated. To obtain some idea of the relative possibilities of the various types of linkages, it has been assumed that the fraction of skin tissue brought into solution by autoclaving has the amino acid composition of gelatin. In Table V are listed the amino acids which have side groups which might react with

TABLE V

Amino Acid Composition of Fraction III, Table I, Based on Amino Acid Composition of Gelatin

	Per cent	Weight mg.	m.mol.
Glutamic acid	5.8	14.9	0.115
Aspartic acid	3.4	8.7	0.076
Histidine	1.0	2.6	0.019
Cystine	0.2	0.5	0.005[a]
Methionine	0.0	0.0	0.0
Tyrosine	0.0	0.0	0.0

[a] Calculated as cysteine.

H and their content in gelatin. The hexone bases have not been included as it is doubtful from the available evidence that side groups bearing a positive charge at pH 7.0 react to any great extent with mustard. Now the total weight of Fraction III given in Table I is 256.8 mg. Using the percentages given, the weight of each amino acid residue in this fraction has been calculated, and in turn its concentration in millimoles. It has then been assumed that all the dialyzable radioactive sulfur in Fraction IIA of Table I has been split off by the alkali from the protein of Fraction III. The total amount of mustard originally combined with Fraction III would thus be 77.5% of 558.3 γ, or 432.7 γ. This corresponds to 0.0027 millimoles of mustard. If it is now assumed that only one of the reactive chlorine groups of mustard reacts with the proteins, then it is obvious that only 1.4% of all the available carboxyl groups furnished by aspartic and glutamic acid can be tied up by this amount of mustard. Likewise, 14% of the histidine or all of the cysteine residues would be needed to react with this quantity of mustard. In view of the fact that this piece of tissue was heavily burned, it can readily be seen that the groups capable of reacting with mustard in this fraction of the skin proteins alone are still far from being exhausted.

The relation of the fixation of mustard in the skin tissue to the production of the lesion is not known. Moritz and Henriques (19) have, however, shown that the severity of the lesion produced in pig skin is proportional to the amount of fixed mustard in the skin. If the fixation of mustard is responsible for the lesion, then the above calculations indicate that the blocking of only a small percentage of the reactive groups in the various proteins is sufficient to cause damage. Such an interpretation finds support in the observations of Davis and Ross (3) that a considerable shift in the O_2 dissociation curve of hemoglobin occurs when only about 6% of the total carboxyl and histidine groups have reacted with H. An alternative interpretation is that all the groups of one protein in particular are susceptible to attack. This seems unlikely. On the other hand, it must not be forgotten that fixation of mustard may play no part in the development of the skin lesion. It is possible that the damage is done by reaction of mustard with small organic molecules which later diffuse away from the site of their damaging action.

SUMMARY

Pig skin treated 24 hr. previously with mustard containing radioactive sulfur was excised and submitted to a fractionation procedure. The various fractions were then analyzed for radioactive sulfur in order to determine the distribution of the "fixed mustard." The results indicate that the major part of the "fixed mustard" is combined with the skin proteins insoluble in weak salt solution. Over half of the combined mustard may be split off by treatment with weak alkali and heat. It is concluded that this fraction is combined with protein carboxyl groups. The type of linkage involved in binding the remainder of the fixed mustard is unknown, but its possible nature is discussed. No evidence could be obtained that phosphate groups are especially concerned in the fixation of mustard by skin tissue. Calculations indicate that only 1 or 2% of the total available skin protein groupings react with mustard, even in skin which has been severely burned.

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A Study of the Conversion of Tryptophan to N′-Methylnicotinamide Using Isotopic Carbon

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INTRODUCTION

It is now generally accepted that tryptophan can substitute for nicotinic acid in the diet of many animals, presumably by causing the synthesis of nicotinic acid. Whether this synthesis occurs as a molecular conversion of tryptophan to nicotinic acid, or by some indirect mechanism, has not as yet been shown.

Using C^{14}, Heidelberger *et al.* (1) have reported that the β carbon of tryptophan is not incorporated into N′-methylnicotinamide. We wish to report data obtained using C^{13} showing that the carboxyl carbon likewise is not incorporated. It seems evident, therefore, that, if the tryptophan molecule is converted into the nicotinic acid molecule, the tryptophan side chain is lost during the reaction.

EXPERIMENTAL

Isotopic tryptophan containing 4.18% excess C^{13} in the carboxyl group was synthesized by one of us as described elsewhere (2). Ten g. of this material was added to 1 kg. of a purified nicotinic acid-free diet (3) and fed to 8 male rats weighing an average of 339 g. Assay (4) of the collected urines showed a total of 648.6 mg. of N′-methylnicotinamide. Using the method of Huff and Perlzweig (5), slightly modified (3), 168 mg. of impure N′-methylnicotinamide picrate, melting about 180°C. (uncorr.) was isolated from this urine. An aliquot of this was then recrystallized repeatedly, yielding 67 mg. of flat yellow leaflets melting at 188–188.5°C. (uncorr.). An authentic sample of N′-methylnicotinamide picrate also melted at 188–188.5°C. (uncorr.) and a mixed melting point showed no depression. Mass spectrometer analysis [1] of this isolated material showed no excess C^{13} to be present.

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[1] All isotope determinations were made on the Consolidated Mass Spectrometer of the National Bureau of Standards. We wish to thank Dr. Fred Mohler and his staff for doing these analyses for us.

DISCUSSION

From the data given here, and the previous report of Heidelberger *et al.* (1), it seems clear that neither the carboxyl nor the β carbons of tryptophan is used by the rat in the synthesis of N′-methylnicotinamide. Since it has been shown previously (3) that urinary N′-methylnicotinamide arises principally from nicotinic acid, it follows that these carbons are not incorporated into nicotinic acid.

This information might be regarded as strengthening the hypothesis advanced by Mitchell and Nyc (6) that the synthesis of nicotinic acid proceeds *via* 3-hydroxyanthranilic acid. This possibility has been tested by Mitchell *et al.* (7), who reported that this compound would substitute for nicotinic acid in the diet of the rat, and by Albert *et al.* (8), who found that this compound increased the urinary excretion of nicotinic acid and N′-methylnicotinamide.

It has been generally believed that tryptophan can increase N′-methylnicotinamide excretion. The evidence for this has been derived principally from chemical estimations, using a reaction (4) which has not been shown to be specific for N′-methylnicotinamide. The isolation, reported here, of this substance in amounts which could have arisen only from synthesis due to the administered tryptophan, provides the first direct evidence to support this proposition. The data of Heidelberger *et al.* (1) cannot be evaluated in this respect due to the very small amounts of N′-methylnicotinamide isolated and to the use of carrier N′-methylnicotinamide.

SUMMARY

N′-Methylnicotinamide has been isolated in substantial amounts after feeding tryptophan.

The carboxyl carbon of tryptophan is not incorporated into N′-methylnicotinamide synthesized by the rat.

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The State of Vitamin A in Eggs [1]

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INTRODUCTION

A survey of the literature failed to reveal reports on the state of occurrence of vitamin A in egg yolk. Therefore, an investigation of this problem was undertaken; the results are reported herein.

METHODS

To overcome the necessity for applying a large correction for the presence of carotenoids in the determination of vitamin A (1,2), the hens from which eggs were obtained were fed a low-carotenoid, all-mash ration compounded from white corn, shorts, oats, wheat bran, meat and bone scrap, soybean meal, fish meal, brewers' yeast,[2] dried skim milk, fish solubles, calcium carbonate, and salt. The ration contained 15% crude protein but only about 300 I. U. of vitamin A from various sources. To each pound of this mash was added 500 γ of riboflavin, 400 I. U. of vitamin D_3, and different amounts of vitamin A, as indicated below.

The hens were White Leghorns in their first laying season. During Period 1, which lasted 3 months, all birds were kept together in a laying house at the college poultry farm and were fed the basal low-carotenoid mash to which was added 1500 I. U. of vitamin A/lb. of ration. At the end of this period, the 9 hens were divided into 3 groups of 3 hens each; they were placed in individual pens in a battery and given the basal mash plus vitamin A supplements administered by capsule. During Period 2, which lasted 3 weeks, hens of Group I received 1000 I. U. of vitamin A alcohol daily, those of Group II received 1000 I. U. of vitamin A ester, and those of Group III received 1000 I. U. of vitamin A daily as carotene.[3] During Period 3, which also lasted 3 weeks, the respective supplements were given at a level of 5000 I. U. daily.

Eggs for analysis were saved at the end of each of the foregoing periods.

Sampling of Egg Yolk

To make possible replicate determinations of vitamin A on the same egg yolk, it was necessary to employ small aliquot samples. The following sampling procedure was found to be satisfactory and overcame several difficulties that otherwise were encountered when attempts were made to weigh small quantities of yolk.

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[1] Contribution No. 378, Department of Chemistry, and No. 180, Department of Poultry Husbandry.

[2] Generously provided by Anheuser-Busch, Inc., St. Louis, Mo.

[3] 10% α- and 90% β-carotene.

The yolk was separated and adhering white was removed by rolling on a muslin towel. The yolk was carefully transferred to a small funnel supported in a beaker, and the combined weight of funnel, beaker, and yolk was obtained. The funnel was placed over a glass-stoppered mixing cylinder, the membrane covering the yolk broken, and the yolk allowed to drain into the cylinder, which contained 10–15 ml. of 15% salt solution.[4] After reweighing residue (essentially yolk membrane), funnel, and beaker, the amount of yolk transferred to the cylinder was determined by difference. Salt solution was added to the cylinder so that each 3 ml. of the final emulsion contained 1 g. of yolk, and the cylinder shaken until the emulsion appeared homogeneous. A moderate amount of foaming occurred but apparently did not introduce an error, since analyses have shown that there was no appreciable concentration of vitamin A in the foam.

Separation of Vitamin A Alcohol and Vitamin A Ester

Six ml. of the yolk emulsion were transferred to a glass-stoppered centrifuge tube; 8 ml. of ethyl alcohol were added and mixed with the emulsion. Thirty ml. of purified Skellysolve B were added and the tube shaken for 3 min. to extract the fat-soluble material. Two additional extractions were made, each time using 15 ml. of Skellysolve B. The 3 extracts were combined in a 250 ml. separatory funnel. Three cold water washings (15–20°C.) of 70 ml., 50 ml., and 50 ml., respectively, were used to remove the alcohol, and each of the wash solutions was extracted successively with 20 ml. of Skellysolve B in a second separatory funnel. The solutions in the two separatory funnels were combined and evaporated to 10–15 ml. under vacuum, using a hot water bath (65°–70°C.) for heating.

An alumina chromatographic adsorption column was prepared as previously described (3). The above-mentioned Skellysolve B solution was added at the top of the column, and the esterified vitamin A was eluted by the flowing chromatogram technique, using 100 ml. of 4% acetone in Skellysolve B. The eluate was evaporated to 1–2 ml. and 2 ml. of water added.

Determination of Vitamin A

Vitamin A was determined by a modification of a method used for blood serum (4). Eight ml. of alcoholic KOH were added to 6-ml. samples of the original egg yolk emulsion (to be used for determination of total vitamin A) and to the chromatographed, evaporated samples (to be used for determination of vitamin A ester), and the samples saponified by refluxing for 20 min. The mixture was extracted in 250 ml. separatory funnels by a modification of the double extraction technique of Boyer *et al.* (5). Thirty ml. of diethyl ether were used for the first extraction and 20 ml. for the second. The extracts in each separatory funnel were washed, first with 60 ml. of cold water,[5] and twice with, respectively, 30 ml. and 20 ml. of cold, acidified, alcoholic wash.

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[4] The purpose of the salt solution was to facilitate separation of solvent layers during the extraction procedure; adding it at this stage prevents yolk from sticking to the cylinder.

After the third washing, the extracts were combined in one separatory funnel; 5–10 ml. of Skellysolve B were added to decrease the solubility of water in the ether layer, and the extracts washed by shaking with 50 ml. of cold water. The solutions were allowed to stand for 15 min. to permit separation of the water, which was carefully drained. The solvents were removed under vacuum, using a hot water bath (65°–70°C.) for heating. The cooled residue was taken up in 10.0–50.0 ml. of Skellysolve F, depending on the concentration of vitamin A in the sample.

Ten ml. of the Skellysolve F solution were transferred to an adsorption tube and the concentration of carotenoid pigments was determined using an Evelyn photometer. The solvent in the tube was evaporated under vacuum and the residue dissolved in 1 ml. of chloroform. The tube again was placed in the photometer, 9 ml. of $SbCl_3$ reagent were added, and the galvanometer reading was recorded at the pause point observed about 5 sec. after adding the reagent. The vitamin A content was calculated in the usual manner, a correction being made for interference of carotenoid pigments, which was small when analyzing eggs from hens on the low-pigment ration.

Content of vitamin A alcohol was calculated by subtracting the values obtained for vitamin A ester content from those for total vitamin A.

In development of the method, it was found by appropriate tests that (a) the extraction procedure removed 97–99% of the vitamin A from yolk emulsions; (b) from solutions of vitamin A alcohol at least 98% of the vitamin was retained by the column after elution with 4% acetone in Skellysolve B; (c) vitamin A alcohol of yolk emulsions recovered from the adsorption column plus vitamin A ester that passed through the column yielded a value 93–106% of that observed for total vitamin A; and (d) on 6 aliquot samples of the same emulsion the average deviation was 1.4% (maximum 2.5%).

RESULTS AND DISCUSSION

Since the hens were given a sub-optimum level of vitamin A during the first period, the increase in vitamin A content of eggs collected at the end of Periods 2 and 3 (Table I) was to be expected (2). It may be noted that, under conditions of this trial, esterified vitamin A was the substance most efficiently assimilated and transferred to the egg. Factors causing the smaller increases when either vitamin A alcohol or carotene was given have not been studied, except that a chromatographic examination revealed that the vitamin A alcohol fed was not entirely homogeneous.

Vitamin A of the yolk was predominantly the alcoholic form, regardless of the form in which it was fed or the level of supplementation. The content of alcoholic vitamin A of individual eggs ranged from 71 to 93% of the total vitamin A (Table I). Eggs from hens receiving alcoholic vitamin A had approximately the same average ratio of the alcoholic to the esterified form of the vitamin at each of the 3 periods (87–90% of the alcohol). Eggs from hens given either vitamin A ester or carotene were of a lower vitamin A alcohol-ester ratio at the third period, when the hens received the larger vitamin supplements. In eggs from the former group, the alcoholic form was 89% of the total vitamin A at the first period, but 76% at the third period; whereas, in eggs from the latter group, corresponding values were, respectively, 82% and 73%. In each of the 3 periods, the average percentage of vitamin A alcohol was lowest in eggs from hens receiving carotene; to what extent the individuality of the hen affected the results is not known.

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[5] If an emulsion forms it may be broken by adding 1–2 ml. of alcohol.

TABLE I

Vitamin A in Eggs from Hens Receiving Supplements of Vitamin A Alcohol, of Vitamin A Ester, and of Carotene

Supplement	Hen no	Total vitamin A, γ/g.			Per cent increase vit. A over previous period		Per cent vitamin A as the alcoholic form		
		Period[a]			Period		Period		
		1	2	3	2	3	1	2	3
Vitamin A alcohol	3966	2.2	3.7	5.6	74	51	90	88	89
	3976	2.2	3.3	5.6	53	69	86	80	88
	3978	1.7	3.6	5.2	112	44	86	93	92
Average		2.0	3.6	5.5	80	55	87	87	90
Vitamin A ester	3974	1.8	6.5	13.9	257	114	90	90	81
	3983	2.1	6.2	13.6	188	121	88	89	76
	3986	1.5	6.2	12.8	300	108	88	88	71
Average		1.8	6.3	13.4	248	114	89	89	76
Carotene	3967	1.9	2.6	6.7	38	154	83	74	71
	3995	1.8	2.8	7.7	53	178	87	84	71
	3994	2.1	3.0	7.1	43	134	77	84	78
Average		2.0	2.8	7.2	45	155	82	80	73

[a] Length of each period and respective levels of supplementation are given in the text.

Work of Parrish *et al.* (4) with cattle, and Hock and Hock (6) and Clausen *et al.* (7) with human subjects, indicated that the percentage of vitamin A ester in the blood increased when large amounts of vitamin A in either the alcoholic or esterified form were fed. Similarly, the percentage of vitamin A ester in eggs increased slightly and the percentage of vitamin A alcohol decreased when high levels of esterified vitamin A or carotene were given to the hen. Only minor differences, however, were noted in the percentages of the two forms of the vitamin in eggs collected at the different periods from hens given vitamin A alcohol.

The ratio of alcoholic to esterified vitamin A in eggs is similar to that reported in normal blood serum (4,6,7) but unlike that of colostrum and milk (3), of calf livers (4), and of fish liver oils (8), in which the principal form is the ester.

Although chromatographic separation indicated that vitamin A of the yolk was principally the alcoholic form, it is possible that it was conjugated with protein in the fresh yolk, and that it was freed in the treatment of the yolk preceding extraction.

The solubility characteristics of the principal form of vitamin A in the yolk, the alcohol, are similar to that of the principal yolk pigments, the carotenols (9,10). Both vitamin A alcohol and carotenols contain hydroxyl groups. Neither esterified vitamin A nor carotene contain hydroxyl groups, and neither is found in appreciable quantities in the yolk. Although the mechanism for deposition of vitamin A in eggs has not been established, it is possible that comparative solubilities of the foregoing forms of vitamin A and of various carotenoid pigments might have some relationship to their relative distribution.

SUMMARY

Methods for determination of content and of state of vitamin A in eggs that had a low carotenoid pigment content were described.

Under conditions of this study, vitamin A fed at high levels was transferred more efficiently to the egg when given as vitamin A ester than when given as either vitamin A alcohol or carotene.

The state of vitamin A was determined in yolk of eggs from 9 hens fed vitamin A in different forms. The vitamin A alcohol contents of the eggs analyzed varied form 71 to 93% of the total vitamin A present. At each of the three periods of the trial, eggs from hens receiving supple-

ments of vitamin A alcohol contained approximately the same average percentage of vitamin A in the alcoholic form. Supplements of either esterified vitamin A or carotene given at high levels seemed to cause a small decrease in the percentage of alcoholic vitamin A of the eggs.

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Phosphorylation of Hexoses by Brain Hexokinase [1]

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The hexokinase reaction (1) occupies a position of special importance, since it represents the initial step in the metabolism of carbohydrates.

(1) Hexose + ATP → Hexose monophosphate + ADP

In addition to this "priming function," the reaction may be involved in the active transfer of hexoses across cell membranes. The latter possibility has been investigated (1,2,3) and interesting relationships between the rates of phosphorylation of various sugars by tissues, and their respective rates of absorption *in vivo*, have been found. A number of workers have observed marked differences in the formation of glycogen from glucose and fructose, respectively, by isolated muscle (4,5). Others have noted differences in the rates of conversion of glucose and fructose to glycogen by hepatectomized animals (6,7). Glucose is glycolyzed much more rapidly than is fructose by skeletal muscle, smooth muscle and tissues from various parts of the central nervous system (8–12). Dickens and Greville (12) found a great deal of variation in the abilities of many different tissues to utilize fructose. Meyerhof and Geliazkowa (13) and Meyerhof (14) have found that brain homogenates utilize glucose more readily than fructose. They found that brain extracts, having much less ATPase activity than the homogenates, utilized both sugars equally well when the system was adequately fortified with coenzymes but the extracts utilized fructose much less readily than glucose when the ATP concentration was reduced to low levels. This was interpreted as being due to a much greater lowering of the affinity of hexokinase for fructose than for glucose by decreased levels of ATP.

The report of Cori and Slein (15) that they were able to obtain two protein fractions from rabbit muscle which are specific for the phosphorylation of glucose and fructose, respectively, indicates that the above cited differences in the metabolism of these sugars may rest on the existence, in the various tissues, of enzymes which are highly specific with regard to the hexose molecule. The hormonal control of hexokinase activity reported by the Cori group (16), indicates that the reaction may be an important key in the regulation of metabolism. It was, therefore, of interest to study the various factors which influence the hexokinase reaction with different sugars. This report also includes some data on the phosphohexokinase reaction.

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METHODS AND MATERIALS

The manometric method described by Colowick and Kalckar (17) was employed. For each mole of phosphate transferred from ATP to hexose, one acid equivalent is liberated and displaces one equivalent of CO_2 from the bicarbonate buffer. Iodoacetate was used to stop the series of glycolytic reactions at the triose phosphate stage. Fluoride was added to inhibit phosphatases. When glucose was used as the substrate, the gas evolved resulted from the hexokinase and phosphohexokinase reactions. The latter reaction was measured separately by using glucose-6-phosphate or fructose-6-phosphate as the substrate.

The reaction mixture, expressed in final molarities, was as follows: potassium ATP 0.002 or 0.004 *M* (sidearm), potassium bicarbonate 0.027 *M*, potassium iodoacetate 0.002 *M*, potassium fluoride 0.053 *M*, magnesium chloride 0.008 *M*, hexose or H.M.P. 0.01 *M*. Variations from this mixture, and the nature and amount of enzyme preparations used are indicated in the appropriate tables and figures. Redistilled water was added to make the final volume 3 ml. The flasks were gassed for 10 min. with a mixture of 95% N_2—5% CO_2 while being shaken on the Warburg apparatus. After an additional 5 min. equilibration period initial readings were made. The ATP was dumped from the sidearm at zero time and readings were then taken at intervals during 1 hr. experimental periods. Experimental values of CO_2 liberation were corrected for CO_2 liberation resulting from ATP hydrolysis in controls containing no hexose.

Beef Brain Extract. Prepared from an acetone powder of the gray matter of beef brains as described by Colowick, Cori and Slein (16).

Dialyzed, Lyophilized Beef Brain Extract. Prepared as above and dialyzed 6.5–7 hr. against 6 changes of cold distilled water with the dialyzing tubing tied to a glass fork rotating on an inclined axis to provide constant mixing. The extract was then lyophilized and the dry powder stored in tightly stoppered flasks in the cold. If this powder is kept at −6°C. in tightly stoppered containers it retains its activity for at least 5 weeks. For use it was taken up in cold redistilled water (4 ml./0.1 g. powder) just before each experiment.

Adenosine Triphosphate (ATP). Prepared from rabbit muscle by the method of Lohmann (18) as modified by Dr. G. T. Cori (private communication). Purity >95%.

Hexose Diphosphate (HDP). Commercial HDP obtained from Schwarz Laboratories and purified by the method of Neuberg, Lustig and Rothenburg (19).

Fructose-6-Phosphate. Prepared from HDP by the method of Neuberg, Lustig and Rothenburg (19).

Glucose-6-Phosphate. Synthesized according to the method of Lardy and Fischer (20).

Mannose-6-Phosphate. Synthesized by a method which is to be published from this laboratory.

RESULTS

Enzyme Concentration

Using glucose or fructose as substrates the activities with the various amounts of the enzyme preparation are directly proportional to the

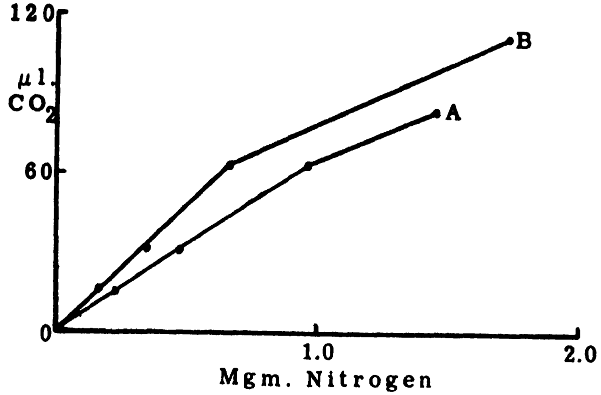

FIG. 1. Effect of enzyme concentration on rate of phosphorylation of glucose and fructose. A = Glucose 0.01 *M*; B = Fructose 0.01 *M*. Activity was measured during the 5–25 min. period. ATP = 0.004 *M*; T = 37°C.

amount of enzyme added within the lower range as shown in Fig. 1. The relationship departs from linearity above levels which correspond to 0.6–0.8 ml. of the enzyme preparation. With fructose as substrate, its concentration becomes limiting at higher enzyme concentrations and contributes to the departure from linearity. In all of the experiments which follow, the level of enzyme extract used was 0.5 ml., which is in the upper portion of the linear range. The graph demonstrates that this technique may be employed for assaying the hexokinase activity of our enzyme extract, and probably that of similar preparations.

Concentration of Hexose

There have been numerous observations of differences in the rate of glycolysis of glucose and fructose by various tissues (8–14), and Meyerhof (13) has shown that, with brain homogenates, increasing the fructose concentration increases its rate of glycolysis nearly to that of glucose. To test the possible difference in the behavior of our system toward these two hexoses, a series of experiments were run in which the ATP level was moderately high (0.002 *M*) in all cases and the concentrations of the sugars were varied. Fig. 2 shows typical results obtained when glucose served as the substrate. At four levels, ranging from 5×10^{-4} to 5×10^{-3} *M*, the respective activities did not vary more than 5% at any time until, at 35 min., the lower level of glucose was, according to claculations, 90% converted to HDP. At this point the reaction practically ceased (point A) while all the others continued at equal rates. The glucose remaining when the reaction ceases (calculated value) cannot be greater than 5×10^{-6} *M*. This shows that hexokinase has a very high affinity for glucose and, as would be expected in such a case, the reaction rate is independent of the concen-

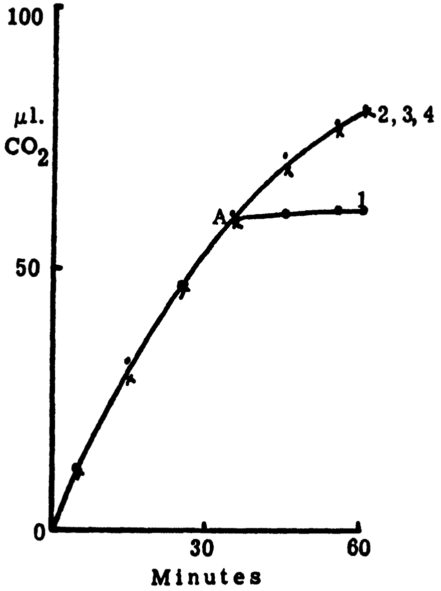

Fig. 2. Influence of glucose concentration on rate of phosphorylation. Initial glucose concentrations: 1 = 5 × 10^{-4} M; 2 = 1.5 × 10^{-3} M; 3 = 3 × 10^{-3} M; 4 = 5 × 10^{-3} M. ATP = 2 × 10^{-3} M. Where one line is plotted for several flasks ● = highest value and x = lowest value recorded.

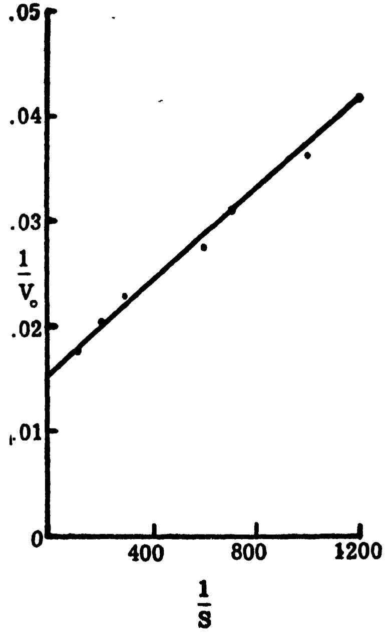

Fig. 3. Determination of K_s for fructose. V_0 = initial reaction velocity in μls. CO_2/10 min. S = substrate concentration in molarity. ATP present at 2 × 10^{-3} M in all cases. Further details in text.

tration of glucose even at the low levels tested. Levels up to 2×10^{-2} M do not show any changes in activity. This situation renders it impossible to measure the Michaelis constant (K_s) for glucose using the manometric technique.

With fructose as the substrate, and ATP at 0.002 M, the activity varied with substrate concentration below 0.01 M. This experiment was repeated with fructose a number of times and the approximate K_s arrived at in the following manner: The increments of CO_2 evolution for each 10 min. period from 5 to 35 min. were plotted (y axis) at the mid-points of the corresponding time interval along the x axis. By extrapolation to zero time the initial reaction velocity (V_o) expressed as μl. CO_2/10 min. was obtained. The values of V_o for each concentration of fructose for all experiments were averaged, and these values used (Fig. 3) to determine K_s by the method of Lineweaver and Burk (21). From this graph the V maximum was found to be 66 μl. CO_2/10 min. and K_s 1.4×10^{-3} M. Since the initial ATP levels were the same in all cases (0.002 M) and not limiting, the differences in initial velocity of reaction are due to the differences in concentration of fructose. Although the technique used measures both the fructohexokinase and the phosphohexokinase reactions we have observed that the latter occurs more rapidly at low concentrations of substrate than does the former. Therefore, the primary phosphorylation of fructose may be assumed to be the rate-determining reaction in this system. While this determination does not meet ideal conditions, these experiments do serve to demonstrate, in a more quantitative fashion than previously shown, that there exists a very marked difference in the affinities of the primary phosphorylating enzyme(s) for these two sugars.

Activity of Various Sugars and Sugar Derivatives

Glucose-6-phosphate and fructose-6-phosphate at levels of 1×10^{-2} M are as rapidly phosphorylated as glucose in this system. When aged enzyme preparations are used, greater activity is obtained with these HMPs than with glucose, which indicates that the hexokinase activity is more labile than the phosphohexokinase. Mannose is phosphorylated 40–60% as rapidly as is glucose, and mannose-6-phosphate is phosphorylated at an intermediate rate. Apparently, the brain preparation can convert mannose-6-phosphate to the corresponding fructose ester.

Beef brain hexokinase did not phosphorylate L-glucose, D-galactose, L-sorbose, potassium D-gluconate, potassium 2-keto-D-gluconate, D-ribose, D-arabinose, L-rhamnose, or D-xylose. When HDP was tested the gas evolution was very low and identical with the controls containing ATP but no hexose. Therefore, the iodoacetate added was effectively blocking the reaction at the triosephosphate stage in this system.

Adding D-mannose, L-glucose, or L-sorbose, at levels of 0.01 M, to flasks containing 0.01 M D-glucose decreased the activity 10–40%. Considering that all three of these sugars produce this effect, and the fact that the decrease in activity was not very great, even at the high levels tested, the effect is not highly specific.

Effects of Various Electrolytes

Racker and Krimsky (22) have reported the glycolysis of glucose by mouse brain extracts to be strongly inhibited by Na^+. Since sodium did not inhibit lactate formation from either glucose-6-phosphate or fructose-6-phosphate they assumed that the inhibitory effect was exerted at the stage of hexose phosphorylation. In yeast, however, Na^+ does not inhibit glucose phosphorylation and K^+ seems to be necessary for the phosphorylation of fructose-6-phosphate to HDP (23). A study was, therefore, made of the effects of these ions on the brain hexokinase and phosphohexokinase reactions.

TABLE I

Effect of Adding NaCl to a Reaction Mixture High in Potassium (0.15 M)

Molarity of NaCl	μl. CO_2/hr. Substrate 0.01 *M* glucose	 Substrate 0.01 *M* fructose
0.0	149	
0.0	153	167
0.005	155	165
0.01	142	158
0.03	129	158
0.06	123	143
0.10	95	153

All reagents including 0.01 *M* phosphate present as potassium salts.

When glucose was the substrate, the activity of the dialyzed beef brain system was increased about 15% by using potassium reagents rather than the corresponding sodium salts. If extra KCl was added up to 0.10 *M*, an irregular and quantitatively insignificant depression of activity resulted with either glucose, fructose, or fructose-6-phosphate as substrate. This is probably a nonspecific effect of salt concentration. When similar additions of NaCl were made, hexokinase activity with glucose was

TABLE II

Effect of Sodium Borate vs. Potassium Borate

Substrate 0.01 *M*	Potassium borate	Sodium borate	μl. CO_2/hr.
	M	*M*	
Glucose	0.01		185
Glucose	0.005	0.005	167
Glucose		0.01	158
Fructose	0.01		113
Fructose	0.005	0.005	112
Fructose		0.01	115
Fructose-6-phosphate	0.01		151
Fructose-6-phosphate	0.005	0.005	143
Fructose-6-phosphate		0.01	149

All reagents of the reaction mixture were potassium salts (0.14 *M*).
Further additions are specifically indicated in the table.

inhibited progressively (Table I). Only a negligible inhibition of fructose phosphorylation occurred. Although it was necessary to add at least 0.01 *M* sodium ion to obtain a definite inhibition this may be due to the relatively high concentration of K^+ in the reaction mixture. Similar results were obtained when sodium borate was compared with potassium borate (Table II), under conditions of constant electrolyte concentration. These results show that the glucokinase activity of the lyophilized dialyzed beef brain is inhibited by sodium ion while the phosphorylation of fructose and fructose-6-phosphate are not.

In other experiments it was found that inorganic pyrophosphate is a strong inhibitor of the phosphorylation of hexoses; 0.015 *M* inhibited 46%; 0.02 *M* inhibited 80%. Since orthophosphate at these same concentrations inhibited only 6% and 14%, respectively, the effect of pyrophosphate is not the result of CO_2 retention. It is possible that pyrophosphate acts by binding Mg^{++} which is necessary for the rereaction. However, additions of $MgCl_2$ up to twice the usual amount added did not reverse the inhibition by pyrophosphate.

Discussion

The difference in the rate of glycolysis of glucose and fructose by various tissues (8–14) may be ascribed to the existence of separate hexokinases which are completely specific for glucose and fructose, respectively, or to a single hexokinase which reacts far more readily with glucose than with fructose. Cori and Slein (15) have presented evidence that, in muscle, these two sugars are phosphorylated by separate enzymes. In the present work, brain extracts were found to phosphorylate glucose and fructose at approximately the same rate. We did, however, notice that certain brain extracts were slightly more active with glucose while others showed greatest activity with fructose. Since the experiments were performed under identical conditions, these differing ratios of activity are presumptive evidence for the existence of two hexokinases in beef brain. Further evidence is afforded by the selective inhibition of glucose phosphorylation by Na^+.

Meyerhof and Geliazkowa (13) found fructose to be glycolyzed more slowly than glucose in brain homogenates. The rate of lactic acid production from fructose could be increased by using a higher substrate concentration or by centrifuging the homogenate to rid it of adenosinetriphosphatase. In direct tests of hexokinase activity in brain extracts Meyerhof and Geliazkowa found glucose and fructose (each at 0.017 *M*) to be transphosphorylated at about equal rates. In the present work we employed similar levels of ATP (0.002 *M*) but much lower concentrations of hexoses. The rate of glucose phosphorylation was found to be independent of sugar concentration down to 0.0005 M. With fructose, however, the rate of phosphorylation decreased as the sugar concentration was decreased below 0.01 M.

Where selective membrane permeability results in slower penetration of tissue by fructose (24) both the decreased quantity of sugar available and the greater K_s for fructose can contribute to the differences in rate of glycolysis of this sugar and glucose.

Furthermore, Meyerhof (14) has shown that glycolysis of fructose is depressed more than that of glucose by decreasing the concentration of ATP to extremely low levels. The manometric procedure employed by us does not lend itself to the accurate determination of hexokinase activity at concentrations of ATP below 0.001 M. A determination of the concentration of ATP required to half-saturate hexokinase in the presence of excess glucose or fructose has, therefore, not been possible. Between 0.001 M and 0.008 M, however, the concentration of ATP did not differentially influence the rate of glucose and fructose phosphorylation.

Further work will be required to determine the nature of the relation between ATP concentration and fructokinase activity.

Much has been written about the possibility of fructose being oxidized by a pathway which does not involve phosphorylation. This speculation was invited by the observation that fructose would support tissue respiration as well as glucose but was far inferior in supporting glycolysis. One must remember however, that a limited quantity of phosphorylated hexose can support 3 times as much respiration (in terms of Q values) as it can glycolysis. If this carbohydrate oxidation can likewise "spark" the oxidation of fatty material present in the tissue (25), then even greater rates of respiration could be obtained.

SUMMARY

1. The preparation of a stable beef brain powder, low in electrolytes, and with good hexokinase and phosphohexokinase activity has been described.

2. The brain preparation phosphorylated glucose, fructose, mannose, and glucose-, fructose-, and mannose-6-phosphates but not galactose, ribose, gluconic acid nor any of a number of other sugars tested.

3. Beef brain hexokinase in the presence of 0.002 M ATP has been shown to have a very high affinity for glucose; K_s = < 0.0001 M. The affinity for fructose is much less; $K_s = 0.0014$ M.

4. Sodium salts inhibited the phosphorylation of glucose but not of fructose or fructose-6-phosphate. This observation, together with the fact that various brain preparations phosphorylate glucose and fructose at differing ratios of activity indicates that two hexokinases with differing substrate affinities may exist in brain as has been demonstrated in muscle (15).

5. Factors affecting differences in the rates of tissue glycolysis and oxidation of glucose and fructose have been discussed.

Footnote Added in Press

Since this paper was submitted, Meyerhof and Wilson (*Arch. Biochem.* **19**, 502 (1948)) reported that fructose is phosphorylated less rapidly than glucose at low substrate concentrations.

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Compounds with "Folic Acid" Activity

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INTRODUCTION

In attempts to assay milk for "folic acid," wide descrepancies in the results secured by the *L. casei* and *S. faecalis* methods were encountered (1). This report is concerned with the activity of compounds suspected of interfering in the "folic acid" assays. It does not explain the discrepancy in the results on milk and does not necessarily invalidate the results on milk or other foods but it does demonstrate: first, that certain compounds have "folic acid" activity; and second, that the activity of at least one of these compounds is a variable when compared to that of pteroylglutamic acid.

PART I

EXPERIMENTAL

The titrimetric microbiological assay methods suggested by Teply and Elvehjem (2) were used in this investigation. The media were modified to include 0.01 ml. of Tween 80 and 0.5 γ of pyridoxamine per tube. An incubation period of 4 days was used unless otherwise noted. These modifications do not offer a major improvement in the methods, but under our conditions the response from assay to assay is somewhat more uniform and the agreement between duplicates slightly improved.

The tests were made over a 100-fold or a 1000-fold concentration range. Tests were made both in the absence and in the presence of pteroylglutamic acid. Two or three different ratios of pteroylglutamic acid and the compound being examined were tested. At least 80 and sometimes 200 or more tubes were used in each test, since all compounds were tested with both organisms.

That thymine has "folic acid" activity has already been shown (3,4,5,6,7). It has been suggested that orotic acid (8) and uric acid (3) have "folic acid" activity. Desoxyribonucleic acid (thymus) was tested because of its thymine content, and 5-methylthiouracil was tested because of the similarity of its chemical structure to that of thymine. Ribonucleic acid (yeast) was tested since it differs from desoxyribonucleic acid in that it does not contain thymine.

The orotic acid was synthesized by the method of Nyc and Mitchell (9). The ribonucleic acid was purified by repeated reprecipitation from acidified ethyl alcohol. The desoxyribonucleic acid was also a purified product. The difficulty of preparing pure nucleic acids have been pointed out by Vischer and Chargaff (10). The compounds used in the present work may not have been pure but they are believed to be reasonably free of pteroylglutamic acid and of thymine in uncombined form. Neither nucleic acid gave any growth response for *S. faecalis* after 18 hr. incubation. After a similar incubation, both free thymine and free pteroylglutamic acid produce a marked growth response with this organism.

DISCUSSION

Under the conditions tested, orotic acid, uric acid, glutamine and ribonucleic acid do not have any stimulatory or inhibitory effect in the "folic acid" assays.

The response shown to 5-methylthiouracil is quite interesting (Table I). At the highest level tested it had no activity for *L. casei* in the absence of pteroylglutamic acid. A stimulatory activity for *L. casei* was shown by 5-methylthiouracil in the presence of pteroylglutamic acid. Even in the absence of pteroylglutamic acid, 5-methylthiouracil was active for *S. faecalis*. One hundred γ per tube gave maximum acid production. More extensive data taken from the entire assay curve show

TABLE I

Possible Interfering Compounds in the Folic Acid Assays

Compound tested	*L. casei* assay			*S. faecalis* assay		
	Highest level tested γ/tube	In absence of pteroylglutamic acid	In presence of pteroylglutamic acid	Highest level tested γ/tube	In absence of pteroylglutamic acid	In presence of pteroylglutamic acid
Purified ribonucleic acid	1,000	0	0	10,000	0	0
Orotic acid (4-carboxyuracil)	500	0	0	5,000	0	0
Uric acid	10	0	0	100	0	0
Glutamine	500	0	0	5,000	0	0
5-Methylthiouracil	1,000	0	+	10,000	+[a]	+[b]
Desoxyribonucleic acid	1,000	+	+	10,000	+	− to +

[a] Very active.

[b] Greater activity shown by combination than by pteroylglutamic acid in one trial.

that pteroylglutamic acid was roughly 28,000 times as active as was 5-methylthiouracil. When combinations of pteroylglutamic acid and 5-methylthiouracil were assayed, the relative activity of the latter compound appeared to be a variable. In one trial, greater acid production was attained with a combination of the two compounds than was attained with high levels of either compound alone. Tests of the combined activity of 5-methylthiouracil and thymine were not made. Strandskov and Wyss (11) found the 5-methylthiouracil inhibited the growth of *L. casei* in the presence of thymine, but exerted no effect in the presence of vitamin B_c supplied as a concentrate from liver.

Desoxyribonucleic acid (thymus) proved to be the most interesting interfering material studied. It showed activity for both organisms both in the absence and in the presence of pteroylglutamic acid. The investigation of the quantitative response to desoxyribonucleic acid proved to be a difficult task and it soon became apparent that the growth response to this compound was a variable affected by two or more independent factors.

PART II

EXPERIMENTAL

Two variables which affect the response to desoxyribonucleic acid are incubation time and the ratio of pteroylglutamic acid to desoxy-

TABLE II

Relation of Incubation Time to "Folic Acid" Response Due to Desoxyribonucleic Acid

Hr. of incubation	Response—interpreted as mγ pteroylglutamic acid/γ nucleic acid. Titrimetric assay. *L. casei*	 *S. faecalis*
16	[a]	0[b]
40	.00046	.0018
64	.00045	.0044
88	.00046	.0046
112	.00056	.0062
136	.00051	.0063
160	.00061	.0063
184	.00063	.0057

[a] For *L. casei*, acid production was too low in the tubes containing both the pteroylglutamic acid and those containing nucleic acid to estimate a response. Marked turbidity was present in both sets.

[b] For *S. faecalis*, pteroylglutamic acid gave both acid production and turbidity, while nucleic acid gave neither in 16 hr.

TABLE III

Relation of Pteroylglutamic Acid: Desoxyribonucleic Acid Ratio to Folic Acid Response of the Latter Compound

Ratios pteroylglutamic acid: nucleic acid in mixture assayed		Average response[a]—interpreted as mγ pteroylglutamic acid/γ nucleic acid	
L. casei	*S. faecalis*	*L. casei*	*S. faecalis*
Pteroylglutamic acid absent		.00047	.00315
1:1,000,000	1:2,000,000	.0017	.00041
1:100,000	1:200,000	.012	.0045
1:10,000	1:20,000	.11	.050

[a] Titrimetric assays. The incubation period was 88 hr.

ribonucleic acid when mixtures of the two compounds are assayed. Data concerning the effect of these two variables are computed in Tables II and III.

DISCUSSION

The effect of incubation time on the response to desoxyribonucleic acid is apparent from the data compiled in Table II. The marked difference in response of *L. casei* and *S. faecalis* is apparent. The data in Table II are expressed in terms of the pteroylglutamic acid which will give an equivalent growth. One might say in another way that, for *L. casei* after 40 hr. incubation, pteroylglutamic acid appeared to be 2,200,000 times as active as desoxyribonucleic acid, while after 184 hr. it appeared to be only 1,600,000 times as active. For *S. faecalis* pteroylglutamic acid appeared to be infinitely more active at 16 hr., 560,000 times more active at 40 hr., and 170,000 times more active at 184 hr.

In the presence of pteroylglutamic acid, a variable response to animal nucleic acid was shown by both organisms. An example of this variation was shown by the data from the experiment designed to study the effect of the pteroylglutamic acid: nucleic acid ratio on the "folic acid" activity of the latter compound (Table III). Expressing these data in another way for *L. casei*, in one case pteroylglutamic acid appeared to be 2,200,000 times as active as desoxyribonucleic acid, in another case it was only 9100 times as active. For *S. faecalis*, in one case pteroylglutamic acid appeared to be 2,400,000 times as active, in another case only 20,000 times as active. These are the results from one assay. Further investigation revealed that there were other unknown variables besides the ratio which affected the response. Since the data are variable it has not been possible to completely interpret the results of assays of all mixtures of pteroylglutamic acid and animal nucleic acid. Very high levels (2–10 mg. per tube) of desoxyribonucleic acid gave partial growth inhibition either in the absence or presence of pteroylglutamic acid. There data, together with those reported in Tables II and III, indicate the error which may arise in attempts to assay mixtures containing both pteroylglutamic acid and animal nucleic acid, when the latter compound is present in significant quantities.

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SUMMARY

Desoxyribonucleic acid and 5-methylthiouracil possess "folic acid" activity for *L. casei* and *S. faecalis*. The relative response to these compounds varies from organism to organism, and, at least in the case of desoxyribonucleic acid, is affected by two or more independent variables.

Under the conditions tested, ribonucleic acid, uric acid, glutamine or orotic acid did not interfere in assays for folic acid.

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Adult Growth in Man and Its Nutrient Requirements

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INTRODUCTION

The nutrition of the adult body may be broken down into (a) the replacement of the basal expenditures of nutrients, the endogenous losses, (b) the provision of nutrients required for adult growth, (c) the provision of nutrients required for muscular activity and muscular work, and (d) supplying the vitamins and other micronutrients required to promote the oxidation of carbohydrates, fats and surplus proteins to furnish energy for (a) and (c). Neglecting the energy requirements for activity, the proper nourishment of the adult relates to the maintenance of the *status quo* of the body, a combination of (a) and (b).

The tissues of the adult body are in a dynamic, not a static, state. They are subject to the irreversible degradation reactions of the endogenous catabolism, the rates of which are not readily affected by activity or food supply. They are also subject to the reversible reactions between tissue and absorbed dietary constituents that Schoenheimer and his associates have demonstrated so clearly by the isotope technic (1). Concerning these reversible reactions occurring in the adult body, Moss and Schoenheimer (2) make the following significant statement:

"It is difficult to regard these interconversion reactions observed in normal animals merely as steps in metabolic degradation. They seem rather to represent automatic and non-interruptable biochemical processes, of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change."

When the intake of nutritive material exactly equals the outgo of the corresponding metabolic end products, and when the food energy consumed exactly equals the energy production in the body (heat, work) plus the unmetabolized energy of the excreta, then the body is said to be in equilibrium with its food supply, and the nutrient requirements corresponding to items (a), (c), and (d) in the nutrition of the adult have been satisfied. But the requirements of adult growth have not.

Growth does not cease with the attainment of adulthood. The morphological aspects of adult growth in man have been demonstrated and discussed by Hrdlička (3), who shows that:

> "The majority of the dimensions or characters, headed by stature, progress perceptibly on the average until well into the fourth decade of life; others, including the skull and the face, increase slightly, in some groups at least, until the fifth or even the sixth decade; and still others, such as the dorsal length and breadth of the nose, the

length of the ears, width of the mouth, depth of the chest, keep on augmenting generally throughout most, if not all, of the adult existence."

The more obvious signs of adult growth are found in the hair and the nails, and, in general, epithelial tissue undergoes mitosis throughout life. Of particular significance is the growth of the skin, which has been described briefly as follows (4, p. 332):

> "The most peripheral layers of the *stratum corneum* consist of dried, horny plates which are constantly being desquamated (*stratum disjunctum*). The cells lost in this way are replaced by new ones which come up from the lower layers. The number of mitotic figures in the deep layers of Malpighian cells corresponds with the intensity of the desquamation of the epidermis at the particular region of the skin."

The quantitative biochemical aspects of adult growth have been little studied. Taylor (5, p. 417) has stated: "A man may lose as much as 100 mg. of nitrogen per day in the growth of the hair," but the supporting data are not given. The general features of hair growth are well described by Danforth (6), but quantitative information on total hair growth is lacking in this review. The importance of the problem resides in its bearing on the nutritive requirements in adult life, because, to the extent that growth occurs in tissues, adequate nutrition will not be represented by nitrogen equilibrium, or equilibrium with respect to other elements, but by positive balances of these elements in proportion to the growth realized.

An attempt has been made to evaluate adult growth in men in terms of nitrogen and phosphorus, using balance data published many years ago from this laboratory in connection with a study of the effects of saltpeter as it occurs in cured meats on the metabolism and health of 23 young adult men (7).

EXPERIMENTAL

The experimental data were secured with 23 university students, ranging in age from 18 to 31 yr., 5 of whom were on an uninterrupted metabolism experiment for 220 days, from December 23, 1907, to July 29, 1908. For the remaining 18 subjects, the metabolism periods ranged from 156 to 212 days, being shortened by late participation in the experiment (subjects 15 and 24) or interrupted by short periods of illness.

The diets during these periods of time were adequate nutritionally and followed a menu extending over 8 days and repeating itself practically unchanged throughout the entire experiment. For an average body weight of about 67 kg., which varied little during the experiment, the men received daily an average of 84 g. of protein (60% from animal foods), 1.46 g. of phosphorus and 3000 calories of metabolizable energy. The individual intakes of nutrients are presented in Table I.

The food consumed by the subjects was weighed and analyzed in 4-day composite samples. Feces and urine were collected quantitatively every 4 days and aliquots analyzed. Nitrogen determinations were carried out by the Kjeldahl method, using mercury as a catalyst and digesting for 1.5 hr. after clearing in the case of food and feces, and for 0.75 hr. in the case of urine samples. Total phosphorus was determined by the gravimetric method, precipitating first as ammonium phosphomolybdate and then as magnesuim ammonium phosphate. The food and feces samples were dry

TABLE I

Description of the Experimental Diets and Changes in Body Weight During the Experiment

Subject	Days on experiment	Average daily diet						Body weights		
		Protein	Fat	Carbohydrate	Fuel value[a]	Phosphorus	Ratio N:P	Initial[b]	Final[c]	Change
		g.	g.	g.	cals.	g.		kg.	kg.	kg
1	212	89	147	352	3087	1.53	9.3:1	70.9	69.3	−1.6
2	204	92	162	432	3554	1.54	9.7	79.4	82.8	+3.4
3	212	81	106	328	2590	1.38	9.4	57.3	55.8	−1.5
4	220	99	194	435	3882	1.66	9.5	78.3	81.3	+3.0
5	212	81	115	353	2771	1.47	8.8	57.0	57.1	+0.1
6	212	78	111	396	2895	1.44	8.6	71.3	70.2	−1.1
7	220	79	146	405	3250	1.46	8.7	68.4	69.3	+0.9
8	220	85	156	329	3060	1.50	9.1	69.1	66.8	−2.3
9	196	83	115	413	3019	1.74	8.0	65.9	62.8	−3.1
10	212	89	129	387	3065	1.44	9.9	76.1	78.9	+2.8
11	212	88	108	412	2972	1.53	9.2	63.6	64.0	+0.4
12	208	91	150	351	3118	1.56	9.3	67.0	64.8	−2.2
13	212	85	108	364	2768	1.34	10.2	67.9	67.4	−0.5
14	208	67	122	358	2798	1.21	9.0	54.6	51.3	−3.3
15	156	87	135	376	3067	1.46	9.5	62.3	68.0	+5.7
16	220	72	120	365	2828	1.12	10.3	63.6	62.1	−1.5
17	212	84	151	328	3007	1.52	8.8	71.6	70.8	−0.8
18	220	94	154	419	3438	1.57	9.6	72.9	72.1	−0.8
19	204	85	122	353	2850	1.43	9.5	66.3	63.8	−2.5
21	212	84	143	307	2851	1.57	8.5	57.9	58.0	+0.1
22	192	85	132	322	2816	1.45	9.1	66.2	62.9	−3.3
23	204	66	94	306	2334	1.11	9.6	57.5	53.8	−3.7
24	156	91	115	372	2887	1.55	9.3	61.3	63.9	+2.6
Average	205.9	84.1	132.0	368.0	2996.0	1.46	9.26	66.36	65.96	−0.41

[a] Estimated by application of the Atwater factors: 4 cal./g. for protein and carbohydrates, and 9 cal./g. for fat.

[b] Average for first 16 day period.

[c] Average for last 16 day period.

ashed; the urine samples were treated by acid oxidation. The phosphorus methods were carefully checked for accuracy.

DISCUSSION

The nitrogen and phosphorus balances of the men, after adjustment to the selected diets in a preliminary period, were positive for the entire

TABLE II

Average Daily Nitrogen and Phosphorus Balances of 23 Adult Men on an Adequate Protein and Phosphorus Diet over Periods of 156 to 220 Days

Subject	Initial age	Initial height	Average weight	Surface area[a]	Nitrogen balance		Phosphorus balance		Ratio of N:P balances
					Per day	Per day/m.²	Per day	Per day/m.²	
	yr.	*cm.*	*kg.*	*m.²*	*g.*	*g.*	*g.*	*g.*	
1	21	172	71.1	1.84	1.26	0.685	0.182	0.099	6.92
2	21	183	80.7	2.03	1.88	0.926	0.226	0.111	8.32
3	31	163	56.6	1.60	1.02	0.638	0.103	0.064	9.90
4	23	188	79.8	2.06	1.70	0.825	0.226	0.110	7.52
5	29	171	57.8	1.68	1.10	0.655	0.153	0.091	7.19
6	22	183	70.4	1.91	1.17	0.612	0.164	0.086	7.13
7	19	176	69.7	1.85	1.60	0.865	0.252	0.136	6.35
8	25	179	68.8	1.86	1.23	0.661	0.160	0.086	7.69
9	21	181	63.7	1.82	1.10	0.604	0.151	0.083	7.28
10	22	170	77.5	1.89	1.66	0.878	0.181	0.096	9.17
11	18	173	64.8	1.77	1.39	0.785	0.177	0.100	7.85
12	23	174	67.6	1.81	1.64	0.906	0.195	0.108	8.41
13	20	179	67.4	1.85	1.24	0.670	0.181	0.098	6.85
14	22	163	53.8	1.57	1.05	0.669	0.139	0.088	7.55
15	28	168	65.5	1.74	2.04	1.172	0.239	0.137	8.54
16	19	179	63.0	1.80	1.10	0.611	0.147	0.082	7.48
17	29	170	70.8	1.82	1.56	0.857	0.181	0.099	8.62
18	19	177	73.3	1.90	1.74	0.916	0.203	0.107	8.57
19	22	173	65.5	1.78	1.18	0.663	0.163	0.092	7.24
21	22	170	58.5	1.68	1.27	0.756	0.198	0.118	6.41
22	26	171	63.6	1.74	1.36	0.782	0.161	0.092	8.45
23	25	165	55.9	1.61	0.91	0.565	0.127	0.079	7.17
24	22	173	62.7	1.75	1.51	0.863	0.118	0.067	[12.80][b]
Average	23.0	174	66.5	1.798	1.379	0.764	0.175	0.0969	7.76
Standard deviation					.302	.146	.0379	0.0182	0.898
Coefficient of variation					21.9	19.0	21.7	18.8	11.6

[a] By the DuBois height-weight formula (8).

[b] Omitted from the average after application of Chauvenet's criterion for the rejection of extreme variates.

experimental period of 156–220 days. These indicated accumulations of nitrogen and phosphorus in the body, averaging 1.38 and 0.175 g./day, respectively, are the resultants of two causative factors, neglecting for the moment the possible existence of constant errors in the analytical determinations. One factor is the loss of these elements through the skin, which was not measured in this experiment in conformance with the usual procedure in determining metabolic balances. When sweating is minimal, these daily dermal losses may be estimated roughly at 360 mg. of nitrogen and 0.5 mg. or less of phosphorus (5,9), leaving about 1 g. of nitrogen and 174 mg. of phosphorus unaccounted for.

The second factor is the retention of nitrogen and phosphorus in the body for adult growth. It should be noted from the data of Table I that these positive balances of nitrogen and phosphorus were not associated with general increases in body weight—in fact quite the reverse—so they can hardly be considered as expressions of adolescent growth.

The daily balances in the table have been reduced to the square meter of body surface on the assumption that the losses of nitrogen and phosphorus in the insensible perspiration, as well as retentions for adult growth, apparently mainly in the growth of epidermal tissue and epidermal structures, would be in proportion to the cutaneous area (10). In support of this assumption, the daily balances of nitrogen and phosphorus for the 23 subjects are appreciably correlated with the estimated surface areas. For nitrogen, the correlation coefficient by the product-moment method of Pearson is +0.551, and for phosphorus, +0.581. Both of these correlations are significant at the 1% level or better (11).

The nitrogen and phosphorus balances are correlated with one another to a highly significant degree, r being +0.742, indicating that they may, to a considerable degree, be serving the same functions in adult growth, such as entering into the composition of the same tissue constituents, or of different tissue constituents, the growth of which is regulated by the same mechanism.

The narrow ratios of nitrogen to phosphorus in the balances listed in the table averaging 7.8 to 1 (5.7 to 1 if correction is made for minimal sweating) are difficult to explain, since the ratio is so much wider in hair (12), about 170 to 1, in skin (13), about 60 to 1, and in the insensible perspiration (14), which has been reported to contain appreciable amounts of nitrogen but no detectable amounts of phosphorus. The striated muscle contains about 23 parts of nitrogen to 1 part of phosphorus and the skeleton 0.63 to 1 (12). It may be surmised that the phosphorus balances involve, to a large extent, secondary or delayed calcification of the teeth, skeleton and cartilage, and perhaps slow accumulations of inorganic and organic phosphates in the soft tissues (15, 16,17). The production of sebum requires phosphorus retention because of the phosphoproteins and phospholipides it contains.

The possibility must be considered that the persistent and considerable positive balances of nitrogen and phosphorus in these young men over prolonged periods of time are artefacts, due to defects in the collection of excreta or in the analysis of foods and excreta. While the questions here cannot be settled definitely, the following comments are pertinent.

The men lived in 2 houses owned by the University and were under constant supervision by University staff members during their residence there. Irregularities in the data of urinary and fecal output were never sufficiently prominent to suggest either that food other than that prescribed was being consumed, or that feces or urine were being incompletely collected. The average apparent digestibility of nitrogen for all men and all periods was 90.6%, and that of ether extract was 97.2%. These values may be compared with those of Atwater and Bryant (18) relating to similar mixed American diets, *i.e.*, 92 for protein and 95 for ether extract.

The creatinine output in the urine per day furnished a rough check on the completeness of collection, suggesting no considerable errors here. The creatinine coefficient for all men and all periods averaged 28.5 mg./kg. body weight, somewhat above the normal range for men of 20 to 26 given by Peters and Van Slyke (19, p. 908).

The nitrogen determinations may be questioned because of the shortness of the period of digestion with sulfuric acid, potassium sulfate and mercury, *i.e.*, 1.5 hr. after clearing for food and feces samples, and 0.75 hr. for urine samples. Longer periods of digestion are advocated by Chibnall, Rees and Williams (20) and by Miller and Houghton (21), and we have found it advisable to extend these periods to 4 hr. for food and feces samples and to 2 hr. for urine samples. But the advantage in the longer digestion period is small (generally 1 or 2% increased titration), and, in the experiment under discussion, would tend to increase, rather than decrease, the indicated nitrogen balance.

SUMMARY AND CONCLUSIONS

The balances of nitrogen and phosphorus of 23 young men subsisting on an adequate diet over a period of 156 to 220 days were consistently positive as ordinarily determined, and averaged 1.379 g. of nitrogen (standard error, 0.063) and 0.175 g. of phosphorus daily (standard error, 0.008) and 0.764 ± 0.030 g. nitrogen and 0.0969 ± 0.0038 g. of phosphorus per $m.^2$ of body surface daily. These balances are positively correlated with the surface areas of the men and with each other.

Dermal excretion of nitrogen in the insensible perspiration with minimal sweating may account for 0.38 g. of N daily, but for very little phosphorus.

The remainder, of approximately 1 g. of nitrogen and 0.174 g. of phosphorus daily, equivalent to 0.56 and 0.10 g., respectively, per $m.^2$ per day, may be considered as being retained for adult growth, relating mainly to the growth of the skin (derma) and the epidermal structures (hair and nails), and, in the case of phosphorus, possibly to a secondary or delayed calcification of the bones and teeth, or to the deposition of phosphates in the soft tissues.

The continuously positive balances of nitrogen and phosphorus observed in this experiment do not seem to be a residuum of adolescent growth, nor an artefact produced by a combination of experimental errors.

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The Effect of Lignin-Destroying Fungi upon the Carbohydrate Fraction of Wood

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INTRODUCTION

Information on the average chain length and the chain-length distribution of the cellulose as it exists in wood and other liquified cellulosic materials is desired for determining to what extent the commercial pulping processes, and the subsequent stages of pulp manufacture, change the original state of these characteristics.

No method is as yet available by which the cellulose or the total carbohydrate fraction can be isolated from lignified cellulosic materials without degradation. Holocellulose, although representing nearly quantitatively the total carbohydrate fraction of the wood, contains the cellulose in a more or less degraded state, even if isolated by the frequently used, and supposedly mild, sodium chlorite method. Investigations of the average chain length and of the chain-length distribution of such preparations show that the original cellulose chain molecules have become shortened to at least the same as, or to an even greater extent than, by a mild sulfite pulping process.

The idea that the cellulose and the non-cellulosic carbohydrates may be removed by biological methods, *i.e.*, by the action of certain fungi, in order to isolate the lignin constituent by milder means than those ordinarily employed, has been advanced repeatedly during the last 20 years. Likewise, it is well known that certain other fungi exert a preferential attack upon the lignin constituent of the wood, leaving a carbohydrate portion as a residue (1).

More recently, this latter fungal activity, also known as "white rot," has been particularly studied by Campbell (2). However, the fungi which Campbell used on a number of different wood species removed only a relatively small portion of the lignin, that is, not more than about 35% with *Polystictus versicolor* on beechwood after an incubation period of 10 weeks (2a), or 27–28% with *Stereum hirsutum* on oak sap wood after 13 months (2b). Nevertheless, it appeared possible that better results might be obtained with other fungi in combination with wood species in which the lignin was more accessible than in those which Campbell employed, and with a longer incubation time.

In his numerous experiments, Campbell had found that the white rot fungi which he used did not confine their attack to the lignin constituent of the wood; invariably, they also, and more or less simultaneously, attacked the carbohydrate portion, and of this, the pentosans to a greater extent than the cellulose. This conclusion was mainly derived from the solubility of the decomposed wood samples in dilute NaOH solution, together with other analytical data.

Campbell's findings suggested a gradual degradation of the cellulose—that is, a gradual shortening of its original average chain length. In view of the objective of the present study, it appeared of interest actually to determine the average chain length of the cellulose component as the lignin was being consumed by the fungi, as well as in the residue eventually obtained.

The following is an account of a preliminary study on the effect of certain fungi upon the carbohydrate fraction of some wood species and other cellulosic materials.

It would be expected that fungi of the genus *Polyporus*, some of which are known to cause white rot, are particularly suitable for a biological removal of the lignin from wood.

The woods used in the present study were in the form of 1 × 2 × 4 in. blocks; these were placed into quart Mason jars, together with sufficient cotton and water to maintain the desired moisture content. The blocks, comprising 9 species of wood—jack pine, white pine, white spruce, black spruce, balsam, larch, chestnut, white birch, and aspen—were inoculated (in one series with *Polyporus anceps* and in another with *P. paragamenus*[1], using the mycelial mats grown on malt agar) and kept at room temperature in the light of the laboratory. After 7 months, some of the blocks showed evidence of white pockets; however, the incubation period was not sufficient to produce very pronounced areas.

After an incubation period of 20 months, the aspenwood block, which had been inoculated with *P. paragamenus*, and the birchwood block, which had been inoculated with *P. anceps*, looked encouraging for further studies; of the two, the aspenwood block was selected for this purpose.

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[1] These fungi (together with a number of other species of lignin destroyers) were obtained through the courtesy of Dr. A. Richards of the Forest Products Laboratory, Madison, Wisconsin.

This block showed 3 main portions which differed in color, indicative of attacks of varying intensity; they were designated as the tan, the pink, and the white portions. The Mäule test indicated lower lignin content for the white and pink portions than for the tan.

The 3 portions, all of which represented fibrous material of the apparently unchanged original wood structure, were separated from the block by means of a razor blade and forceps. The samples were washed with water (with intensive stirring) to remove the mycelial material as far as possible, dried in the air, and ground in a Wiley mill, using a 20-mesh screen. They were analyzed for lignin, pentosan, and nitrogen, the latter determination being made to obtain an idea of mycelial material retained by the samples.

In the determination of the degree of polymerization (D.P.)—*i.e.*, the molecular weight divided by 162, the molecular weight of the individual unit of the cellulose chain molecule—of the three samples, an attempt was first made to use the cuprammonium hydroxide method for ascertaining the specific viscosities from which to calculate the D.P.'s, using the Staudinger function (3). However, the samples were only partly soluble in this solvent, even after being allowed to stand for about 2 weeks.

The nitration method[2] (as used in previous studies on chain length and chain-length distribution (4) and since then improved in various respects) was then tried and, although more or less of the nitrated material was insoluble in acetone, the amount could be determined by centrifuging the dispersion and weighing the residue. From the viscosity data for the soluble portions, an approximate idea of the degrading attack of the fungi upon the carbohydrate fraction was obtained.

The analytical results, the amounts of acetone-soluble material in the nitrated material, and the average D.P.'s of the soluble portions are shown in Table I.

On the assumption that the three portions of the wood represent progressive stages of attack, the data show that *P. paragamenus* removed increasing quantities of lignin from the wood; the greatest effect was observed in the white portion, the lignin content of which was only 3.4%, as compared with 17.5% for the original wood. It is probable that with a longer period of incubation, the lignin would have been removed almost completely.

However, the attack of the fungus upon the wood was not confined to the lignin. It is seen that the pentosan content also decreased consi-

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[2] Briefly, this method consists of converting the cellulosic materials into a derivative which is soluble in an organic solvent and of which the viscosity can be determined. It has been shown that the conversion of the cellulosic materials into their nitrates proceeds practically without degradation of the cellulose if certain precautions are observed. Almost all the non-cellulosic carbohydrates and the lignin become soluble upon nitration and subsequent purification of the nitrate. The final product, therefore, is almost entirely cellulose nitrate.

TABLE I

Effect of P. paragamenus upon Aspenwood

Portion of wood block	Lignin[a]	Pentosans[a]	Nitrogen[b]	Acetone solubility of nitrate	D.P. of soluble portion[c]	Cellulose (calculated)
	per cent	*per cent*	*per cent*	*per cent*		*per cent*
Original	17.5	19.3	0.06	100	1600	60.68
Tan	10.4	12.8	0.50	70.6	705	73.84
Pink	4.5	8.3	0.54	92.1	587	84.20
White	3.4	8.4	0.42	79.7	444	85.32

[a] Lignin and pentosans were determined according to Institute standard methods.

[b] Determined by Kjeldahl.

[c] Calculated for cellulose.

derably, 56.6% of the original pentosan material having been destroyed in the white portion at the end of the incubation period.

On the other hand, the cellulose content of the samples increased, if calculated by subtracting the sum of lignin, pentosans, nitrogen, and resins (determined by extracting the original wood with a 50:50% mixture of benzene and alcohol, and amounting to 2.46% of the oven-dry wood) from 100. Since these increases in cellulose content were calculated from the actually determined data—that is, leaving out of consideration acetyl and uronic acid contents which might amount to 7–8%—the actual cellulose contents would be correspondingly lower. In this preliminary study, these two values were not determined and, therefore, the comparison of the composition of the recovered materials with that of the original wood gives only an approximate idea.

As is seen from Table I those portions of the carbohydrate materials which were left in the fungi-treated samples were considerably degraded even if the average D.P. were corrected for both lignin and pentosan contents.

The D.P. of 1600, ascribed to the original aspenwood in Table I, refers to its holocellulose content. The holocellulose was isolated from the wood by the sodium chlorite method according to Wise (5), and its average D.P. was determined by the nitration method.

Because of the limited acetone solubility, the average D.P.'s of the nitrates from the inoculated samples do not represent the true values of their cellulose constituent. However, even if the acetone-insoluble portions contained material of higher D.P.'s than the soluble portions, the average results would still reveal considerable degradation.

It is probable that the partial acetone insolubility of the inoculated samples resulted from the presence of some mycelial material as indicated by the nitrogen contents of these samples (these were 8–9 times that found in the original wood). The literature (6) on the chemical composition of fungi contains information from which it would appear that the proteinaceous constituents and, perhaps to an even greater extent, the higher fatty acids and glyceride constituents of the fungi are responsible for their resistance to the action of acids, alkalies, *etc.*, and, particularly, to cellulose-dissolving agents, such as cuprammonium hydroxide. Possibly this behavior would explain the pronounced insolubility in this solvent, even of the white sample, and it may also explain the difficulties encountered in preparing a completely acetone-soluble nitrate. That incomplete acetone solubility is probably also a result of incomplete nitration is borne out by the fact that the yields of nitrated material were rather low, as well as by the negative result of an attempt to nitrate a sample of pure culture mycelial material. The nitration acid—which was of the same composition as that used for the nitration of the inoculated wood samples—did not penetrate the mycelium; the latter floated on the surface of the acid.

Since the mycelium penetrates the whole fibrous structure thoroughly, it is difficult, if not impossible, to separate it from the carbohydrate material, unless methods are employed which would destroy the latter. On the other hand, the quantity of mycelial material retained would seem to have been too small to change the ratio between lignin and carbohydrates in the treated wood samples to any marked extent.

As pointed out by Campbell (1) the effect of the fungal action would seem to be comparable with that of a gradual hydrolysis *in vitro*—for example, by acids. If the hydrolysis lasts long enough, all chain molecules are shortened, eventually to yield oligosaccharides, cellobiose, and glucose. The decrease of the original D.P. to a value of as low as 444 for the white sample indicates an intermediate stage.

Further, it appeared to be of interest to throw some light upon the question of to what extent the degradation of the cellulose component of the carbohydrate fraction in the wood was effected by the simultaneous presence of either the lignin or the pentosans (non-cellulosic carbohydrates) or of both these components.

For this purpose, isolated cellulose (purified cotton linters) and holocellulose (prepared from aspenwood by the Wise method, see above), after sterilization in an autoclave at 15 lb. pressure (220°C.) for 20 min., were inoculated with *P. paragamenus* (grown on malt agar) in Erlenmeyer flasks. Czapek-Dox solution was used as the growth-assisting medium but the glucose constituent of the solution was separately added to the inoculated samples. Two additional inoculated samples were allowed to stand without glucose being added. Further, blank tests were run to ascertain the D.P.-decreasing effect of autoclaving upon the two cellulosic materials. The incubation periods in all cases were 9 months.

At the end of this period, the unconsumed malt agar was removed as far as possible with the aid of a pair of forceps. The samples were then thoroughly washed with hot and cold water, dried in a vacuum oven at 60°C. for 4 hr., and subjected to average D.P. determinations by the nitrate method.

The results, together with essential experimental details, are shown in Table II.

TABLE II

Effect of P. paragamenus upon Cotton Linters and Holocellulose from Aspenwood

Sample	Treatment	Appearance	D. P.	Acetone-insoluble
				per cent
Cotton linters[a]	Sterilized, inoculated; no glucose	Little or no growth	1145	—
Cotton linters[a]	Sterilized, inoculated; 0.5% glucose	Little or no growth	1205	—
Holocellulose[b]	Sterilized, inoculated; no glucose	Growth apparent	800	4.5
Holocellulose[b]	Sterilized, inoculated; 0.5% glucose	Growth apparent	1020	15.5
Cotton linters[a]	Sterilized only	Unchanged	1325	–
Holocellulose[b]	Sterilized only	Unchanged	1270	2.0

[a] D.P. of original cotton linters was 1450 (nitrate method).

[b] D.P. of original holocellulose was 1600 (nitrate method).

It is seen that, although little or no growth was apparent, the D.P. of the cotton linters had decresaed. Corrected for the effect of the autocalving alone, the decrease in D.P. with no glucose present amounted to 180 points (from 1325 to 1145), which is slight but distinctly more than could be considered as experimental error between two tests on the same sample. Under otherwise equal conditions but with glucose present, the cotton linters were even less attacked, the decrease in D.P. amounting to only 120 points (from 1325 to 1205). It wolud thus seem that glucose is a more easily accessible carbon source for the fungus than cellulose and, therefore, protects the latter.

The pronounced growth which was apparent on the holocellulose preparation was reflected in the considerable decrease in D.P. Although autoclaving alone was more detrimental to the holocellulose (the D.P. decreased from 1600 to 1270), the growth of the fungus caused a further decrease of 470 points—*i.e.*, from 1270 to 800. Again, the presence of glucose reduced this attack to 250 points—*i.e.*, the D.P. dropped only from 1270 to 1020.

It is seen from the last column of Table II that the cotton linters nitrates were completely soluble in acetone, Obviously, if any mycelial material was retained by the treated samples, it was not sufficient to interfere with the nitration procedure. However, in the case of the holocellulose preparations (which were much more attacked), difficulties in producing completely soluble nitrates were again encountered. That the insoluble portion was greater for the sample which appeared less attacked may probably be explained by assuming a greater production of mycelial material from the glucose. Part of the insolubility of the holocellulose nitrates from the inoculated holocellulose preparations resulted from the limited solubility of the nitrate from the untreated sample (2%). Such insolubility has been frequently observed with holocellulose nitrates and, although it is obviously connected with their non-cellulosic carbohydrate contents, it has not yet been satisfactorily explained.

The pentosan content of the treated holocellulose samples was not determined. However, it would seem safe to assume that considerable amounts of it were destroyed by the fungus, as was the case with the aspenwood. The rather low D.P. of 800 for the holocellulose inoculated without the addition of glucose indicates considerable degradation of its cellulosic constituent.

It is probable that the attack of the fungus upon the cotton linters and the holocellulose would have gone further, if the incubation period had been as long as that for the wood samples—*i.e.*, 20 instead of 9 months.

Thus, it may be concluded that pure cellulose is only slightly attacked by a lignin-destroying fungus, that the presence of pentosans in a cellulosic material (such as holocellulose) gives rise to considerably intensified attack upon the cellulose constituent and that, in wood, both the pentosans and the lignin would seem to favor the attack of the fungus upon the cellulose constituent.

Nevertheless, with the results of this preliminary study, the biological methods of isolating the cellulose constituent from lignified plant tissue without degradation are by no means exhausted. Apart from the possibility that more suitable microoragnisms might be found, the use of specific and purified enzymes would seem to be promising. Some thought might also be given to the possilility of stabilizing the carbohydrate portion of the wood against fungal or enzymic attack by the introduction of certain substituents. Such treatment must, of course, leave the lignin unchanged—*i.e.*, must not prevent the fungal attack of the lignin or any modification of the latter.

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Structural Requirements of Specific Substrates for Chymotrypsin. I. The Contribution of the Secondary Peptide Group[1]

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INTRODUCTION

All the known specific substrates for chymotrypsin which are split at a rapid rate possess two common chemical groupings. The first of these is the bond which is susceptible to enzymatic hydrolysis, whether it be a peptide, amide, or ester bond, and involves the carbonyl group of either tyrosine or phenylalanine residues (1–5). The second common grouping is the secondary peptide bond which involves the imino group of these aromatic amino acid residues. In the present paper an attempt is made to evaluate the contribution of the secondary peptide bond of specific substrates to combination with, and hydrolysis by, chymotrypsin, whereas, in a companion paper (6), a more general analysis of the structural requirements of specific substrates for chymotrypsin will be presented.

In the work presented herein, various compounds were prepared in which the secondary peptide group was replaced by other groupings, or else entirely omitted. The influence of chymotrypsin on these compounds was investigated and interpreted on the basis of kinetic analyses previously described (7). It has already been shown that one of the most rapidly split substrates for chymotrypsin is benzoyl-L-tyrosine ethyl ester (4, 5). However, because of the relative ease of preparing analogs of benzoyl-L-phenylalanine methyl ester, this compound was chosen for the standard of comparison.

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[1] Part of a thesis to be submitted by John E. Snoke to the Graduate School of Duke University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Predoctorate Fellow of the U. S. Public Health Service.

EXPERIMENTAL

Enzyme

The preparation of chymotrypsin was the same as that previously used (8).

Substrates

Benzoyl-L-Phenylalanine Methyl Ester (BPME) was prepared by benzoylating L-phenylalanine (9), and esterifying with absolute methanol and HCl as a catalyst. After recrystallization from ethanol-water mixture, m. p. 81°C. Calculated: N, 4.95%; Found: N, 4.85%.

Methyl-l-β-phenyllactate (l-MPL). The preparation of this compound was described by McKenzie and Martin (10), who prepared β-phenyllactic acid by conversion of α-bromo-β-phenylpropionic acid to the corresponding hydroxy acid, followed by chemical resolution of the optical isomers, and esterification with HCl as catalyst. For the preparation of small amounts, we have found it more convenient to prepare the optically active hydroxy acid directly from L-phenylalanine, following the procedure of Dakin and Dudley (11). Esterification was accomplished by refluxing for 6 hr. a solution of 5.0 g. of the acid in 50 cc. of commercial absolute methanol in the presence of a few cc. of concentrated H_2SO_4. After removing the excess of methanol by concentration *in vacuo*, the oily residue was taken up in ether, and the ether solution washed successively with water, saturated $NaHCO_3$, and again with water. After removal of the ether, the ester was distilled, and the fraction boiling at 123–126°C. at 9 mm. pressure was collected. Yield, 3.8 g. of a crystalline solid; m. p. found, 45–46°C.; reported (10) 45.6–46.5°C. Optical rotation (3% in. benzene), $[\alpha]_D = -11.9°$; reported (10) − 12.1°. The D- and DL- forms of this ester were prepared in the same manner.

Methyl-dl-α-Chloro-β-Phenyl Propionate (dl-MCPP). To a cooled suspension of 15 g. of DL-phenylalanine in 300 cc. of water was added 30 cc. of ethyl nitrite. Immediately thereafter, 90 cc. of concentrated HCl was added with vigorous shaking. The resulting solution was shaken in an ice-bath for 1 hr. and then allowed to stand overnight at room temperature. On standing, the evolution of gas was noted, with the simultaneous formation of an oil. The product was extracted with ether, and the ether solution washed with water. The ether was removed *in vacuo* and the resulting oily acid distilled. The fraction boiling at 137–140°C. at 1 mm. pressure was collected. Yield, 9 g. of a light yellow oil which crystallized on standing; m. p., 44°C. $C_9H_9O_2Cl$ (184.6) Calculated: C, 58.6; H, 4.92; Cl, 19.2; Found: C, 59.2; H, 5.05; Cl, 18.0.

Esterification was carried out by refluxing for 6 hr. a solution of 4.0 g. of the acid in 100 cc. of methanol in the presence of 5 cc. of concentrated H_2SO_4. The product was worked up as above and distilled. The fraction boiling at 110–111°C. at 4 mm. pressure was collected. Yield, 3.9 g. $C_{10}H_{11}O_2Cl$ (198.65) Calculated: C, 60.5; H, 5.60; Cl, 17.8; Found: C, 61.2; H, 5.73; Cl, 17.2.

Methyl Hydrocinnamate (MHC). Hydrocinnamic acid (Eastman) was esterified in the manner described above. The ester was distilled and the fraction boiling at 236–238°C. at atmospheric pressure was collected.

Methods

Enzymatic experiments were carried out at pH 7.80 in the presence of 0.01 *M* phosphate buffer at 25°C. Because of the relative insolubility of the substrates in aqueous solutions, all experiments were conducted in the presence of 20% methanol (5). The substrate was weighed out before each experiment, dissolved in 2.0 cc. of methanol, and buffer and water added so that, after the addition of the enzyme solution, the total volume would be 10 cc. The course of the reaction was followed by direct electrometric titration as previously described (8). Enzyme solutions were made up daily by weighing out the appropriate amount of solid, and dissolving it in 0.004 *M* HCl. Enzyme N concentrations were determined either by the semimicro Kjeldahl method or else by measurements in a Beckman ultraviolet spectrophotometer from a standard calibration curve at 282 mμ, which was established by enzyme N analysis.

RESULTS

Benzoyl-L-Phenylalanine Methyl Ester (L-BPME)

Quantitative rate measurements for the hydrolysis of BPME in relation to initial substrate concentration are shown in Fig. 1. Because of the uncertainty of the initial substrate concentration, a_0, in electrometric esterase titrations, this quantity was determined from the slopes of the curves shown in Fig. 1 by a method previously described (8). At each substrate concentration, the course of hydrolysis followed first order reaction equations; however, the first order reaction constant increased with decreasing substrate concentration, as previously described for other enzyme-substrate systems (5, 7). Accordingly, the data were interpreted in terms of an enzyme-substrate dissociation constant, K_m, and the specific reaction constant, k', where $K_m = (k_2 + k')/k_1$, and $k' = V_{\max}/e$.[3]

In order to determine K_m and k', the data were plotted according to one of the equations of Lineweaver and Burk (12), *i.e.*,

$$\frac{a}{v} = \frac{K_m}{V_{\max}} + \frac{1}{V_{\max}}\,a. \tag{1}$$

A plot of this equation for the hydrolysis of BPME by chymotrypsin is shown in the inset graph of Fig. 1, where v was taken as the moles of

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[3] For definition of these symbols, see Ref. 7. Enzyme concentration, e, is expressed as mg. of enzyme N/cc., and hence, the units of k' are $\frac{\text{moles hydrolyzed/min.}}{\text{liter} \times \text{mg. N/cc.}}$. Since a, the initial substrate concentration is expressed as moles/l., whereas v, the initial reaction velocity has been expressed herein as moles/10 cc. (the reaction volume)/min., k' was calculated merely by multiplying $V_{\max}/e$ by 100.

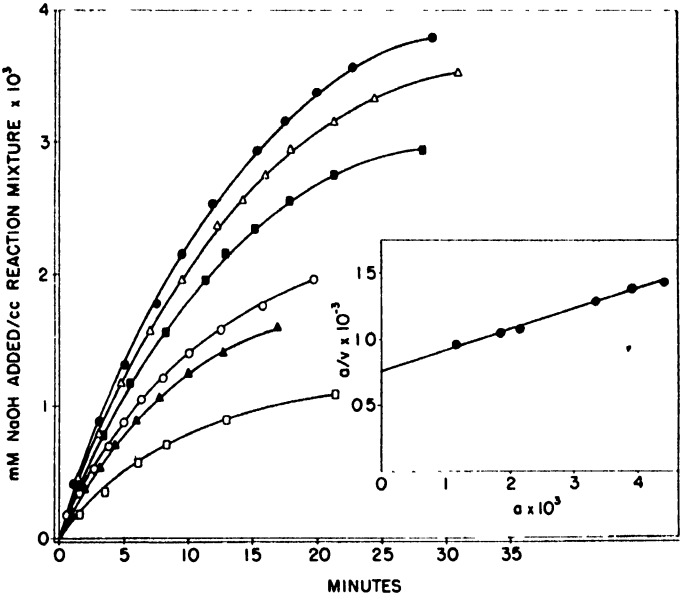

FIG. 1. Hydrolysis of L-BPME at different initial substrate concentrations in 20% methanol, 0.01 M phosphate buffer, pH 7.80, at 25°C. The amount of chymotrypsin N present in these systems (volume 10 cc.) was 0.00115 mg./cc. The initial concentration of BPME is shown by: ● = 0.00440 M, △ = 0.00390 M, ■ = 0.00333 M, ○ = 0.00215 M, ▲ = 0.00185 M, □ = 0.00117 M. The inset graph is a plot of Eq. (1) for the determination of K_m and V_{max}.

substrate/10 cc. hydrolyzed/min., calculated from the initial 2.5 min. of hydrolysis. The calculated values were, $K_m = 0.46 \times 10^{-2}$, and $k' = 0.55$.

Methyl-β-Phenyllactate (MPL)

Exploratory measurements on this and the following substrates revealed that the rate of hydrolysis was of considerably lower order of magnitude than the rate of hydrolysis of BPME. For this reason it was necessary to establish the fact that hydrolysis of these substrates was due to chymotrypsin, and neither due to spontaneous hydrolysis nor caused by the catalytic activity of other enzymes that may have been present as impurities in the crystalline preparation of chymotrypsin.

Of all the substrates used in this work, only MCPP was spontaneously hydrolyzed. However, the rate of spontaneous hydrolysis was only 1/200th that of the enzymatic hydrolysis and, accordingly, too slow to exert any measurable effect on the results of kinetic measurements which never exceeded 120 min., and usually were completed within 35 min. The most likely enzymatic impurity to be suspected in chymotrypsin, is trypsin. All substrates reported herein were tested for tryptic hydrolysis (20 mg. of trypsin/10 cc. reaction mixture), with essentially negative results throughout.

The hydrolysis of *dl*-MPL was unusual, in that the initial substrate concentration, calculated from the properties of the rate curves, was higher than one-half of the analytical concentration, suggesting that the *d*-isomer was hydrolyzed as well, at least in part. Also, the rate of

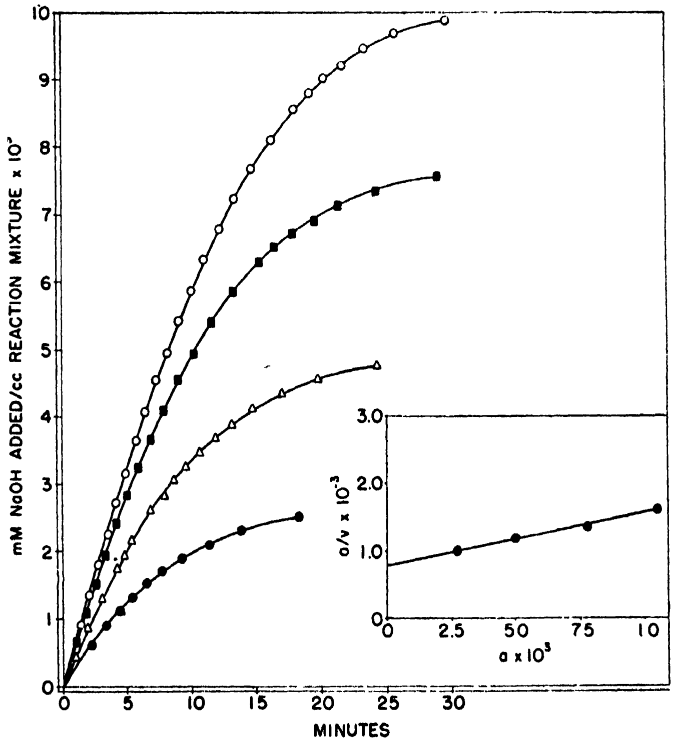

FIG. 2. Hydrolysis of *l*-MPL at different initial substrate concentrations. Experimental conditions as described in the legend to Fig. 1. The amount of chymotrypsin N present in these systems (volume 10 cc.) was 0.0884 mg./cc. The initial concentration of *l*-MPL is shown by ○ = 0.0105 M, ■ = 0.0078 M, △ = 0.0050 M, ● = 0.0027 M. The inset graph is a plot of Eq. (1) for the determination of K_m and V_{max}.

hydrolysis was greatly reduced after about 50% of the racemate was hydrolyzed, indicating different rates for the two enantiomorphs. To substantiate this conclusion, kinetic measurements were performed separately on the two optical isomers as well as on the racemate of MPL. Fig. 2 shows the results of rate measurements on l-MPL at 4 different initial substrate concentrations, while in the inset graph of Fig. 2, a plot of Eq. 1 for the determination of K_m and k' is given. The calculated values were, $K_m = 1.0 \times 10^{-2}$, and $k' = 0.0145$. The corresponding values, determined in the same fashion for *d*-MPL from the plot shown in the bottom diagram of Fig. 3, were $K_m = 3.5 \times 10^{-2}$, and $k' = 0.0024$. It was of interest to note that the rate constants for *dl*-MPL, when calculated on the basis of the initial analytical concentration of *l*-MPL in the racemate, were exactly the same as those obtained for the *l*-isomer alone, indicating that, in a mixture of both isomers, the initial rate-determining step is that of the splitting of the *l*-isomer. This is to be expected from the constants given above, since

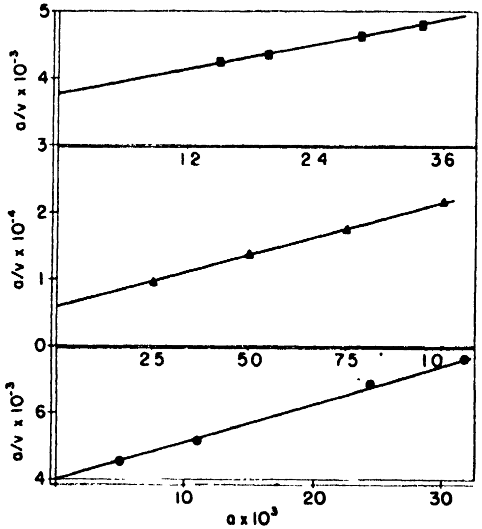

FIG. 3. Plot of Eq. (1) for the determination of K_m and V_{max} for the following substrates: ■ *dl*-MCPP, ▲ MHC, ● *d*-MPL. The amount of chymotrypsin N in these systems (volume 10 cc.) was 0.133 mg./cc. for *dl*-MCPP, 0.305 mg./cc. for MHC, and 0.359 mg./cc. for *d*-MPL.

the affinity of the *d*-isomer for the enzyme is considerably less than that of the *l*-isomer.[4]

The enzymatic nature of the hydrolysis of MPL was established by kinetic measurements at constant substrate and varying chymotrypsin concentrations. Rate constants, k', were calculated for each experiment with the integrated Michaelis-Menten equation (Eq. 14 of Ref. 7), K_m having previously been determined (*vide supra*). In the presence of 4.77×10^{-2} mg. of enzyme N/cc., k' was 0.0147, up to about 50% of hydrolysis of 0.01 *M dl*-MPL, and approximately the same constant was obtained ($k' = 0.0146$) when the enzyme concentration was doubled and tripled.[5]

Methyl-dl-α-Chloro-β-Phenylpropionate (dl-MCPP)

This substrate, too, was hydrolyzed by chymotrypsin at an extremely slow rate. However, controls for spontaneous hydrolysis and tryptic hydrolysis, both of which were noncontributory under the conditions of the experiment, indicated that the observed hydrolysis was caused by chymotrypsin only. At constant substrate concentration, first order reaction kinetics was observed to over 90% of hydrolysis of the racemate. This is in contrast to analogous measurements on *dl*-MPL, and may be taken as evidence that both optical isomers of MCPP are hydrolyzed by the enzyme at the same rate. A plot of Eq. (1) for the determination of K_m and k' of *dl*-MCPP is shown in the top section of Fig. 3. This, as well as the two other plots shown in Fig. 3, were obtained from measurements analogous to those illustrated in Figs. 1 and 2 for L-BPME and *l*-MPL. The calculated values were, $K_m = 1.2 \times 10^{-2}$, and $k' = 0.0023$.

Methyl Hydrocinnamate (MHC)

Calculation of K_m and k' was made in exactly the same fashion already described for the other substrates used in this work. A plot of the data according to Eq. (1) is shown in the center section of Fig. 3. The calculated values were, $K_m = 0.39 \times 10^{-2}$, and $k' = 0.0002$.

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[4] As previously stated (7), K_m contains explicitly the rate of decomposition of the enzyme-substrate complex, k', and hence, is not an unambiguous measure of the affinity of the substrate for the enzyme. However, it is evident from the present data for K_m and k' that the affinity of the *d*-isomer for the enzyme must be considerably less than that of the *l*-isomer of MPL. Thus, $K_m{}^D$ is 3.5 times higher than $K_m{}^L$, whereas k'^D is only about ⅓th of k'_L, K_m being defined as heretofore as $(k_2 + k')/k_1$.

[5] Preliminary experiments revealed that trypsin splits methyl-l-α-hydroxy-δ-guanidinovalerate at one-half the rate of the analogous substrate benzoyl-L-arginine methyl ester. Thus, the ability of the hydroxy group to replace the secondary peptide group of trypsin substrates is even greater than for chymotrypsin substrates.

A summary of the kinetic constants for the hydrolysis of these esters by chymotrypsin is given in Table I.

TABLE I

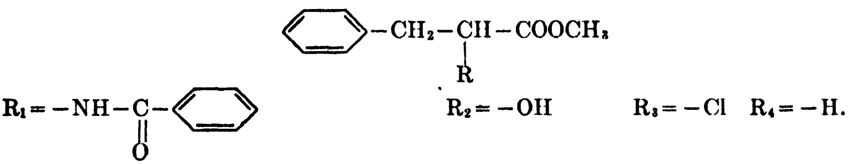
Kinetic Constants for the Hydrolysis of Specific Ester Substrates by Chymotrypsin

Substrate	K_m	k'	C_{max}
	10^{-2} molar		
Benzoyl-L-phenylalanine methyl ester	0.46	0.550	52
Methyl-*l*-β-phenyllactate	1.0	0.0145	0.63
Methyl-*d*-β-phenyllactate	3.5	0.0024	0.03
Methyl-*dl*-α-chloro-β phenylpropionate	1.2	0.0023	0.083
Methyl hydrocinnamate	0.39	0.0002	0.022

To facilitate comparison with previous data on other substrates for chymotrypsin (1–5), maximum values for proteolytic coefficients, C_{max}, are also included in Table I. This constant denotes the maximum value for the first order reaction constant per mg. of enzyme N per cc. which would be observed if the substrate concentration were sufficiently low to render enzyme-substrate combination the rate determining step.[6]

Discussion

The four substrates which have been studied in this work differ from each other only in the nature of the substituent on the α carbon atom of the compound methyl hydrocinnamate, as shown by the chemical structures given below:



The substituent, R, contains a peptide group in BPME, whereas in MPL, R is a hydroxyl group, in MCPP a chlorine atom, and in MHC merely a hydrogen atom. The influence of this residue on the susceptibility of the resulting compound to enzymatic hydrolysis may be 2-fold. The residue may affect the affinity of the substrate for the enzyme, or it may influence the rate of activation[7] of the enzyme-substrate complex as expressed herein by the rate constant k'. All specific peptide, amide and ester substrates for chymotrypsin previously described (1–5) contain a secondary peptide group adjacent to the α carbon atom of the typical amino acid residue. The importance of this structural element for substrates of another proteolytic enzyme is demonstrated by the fact that the interposition of one methylene group between the α carbon atom and the secondary peptide group in substrates for carboxypeptidase, decreases the rates of hydrolysis of the resulting compounds some 1000-fold (13).

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[6] The relation between K_m, k' and C_{max} is (7) $C_{max} = \frac{k'}{2.3K_m}$.

The character of the secondary peptide bond is such that it could enhance combination with the enzyme by hydrogen bonding. If this were the sole function of this group, the rate of activation of the substrates which are devoid of this configuration (MPL, MCPP, and MHC) should be approximately the same as that of the parent compound, BPME, and the respective enzyme-substrate dissociation constants, K_m, should be increased. This is in contrast to the experimental data (Table I), which show that the rates of activation of the compounds containing R_2, R_3, or R_4 are *lower* by two or three orders of magnitude than that of the reference compound BPME (R_1). This is even true for MPL which contains, in place of the secondary peptide bond, a hydroxyl group which is likewise capable of forming hydrogen bonds.

The lower rates of activation of the substrates which are devoid of the secondary peptide bond suggest that this bond influences the susceptibility of the substrate to hydrolysis after combination with the enzyme has taken place. In the absence of thermodynamic data[8] it is difficult to elucidate the mechanism whereby the susceptibility is decreased; however, it seems reasonable to assume that the secondary peptide bond serves as a point of attachment, and that the loss of

[7] It is recognized that, in the case of activated adsorption, the adsorption step itself contributes to the activation process, and that the two steps cannot be as discretely separated as is implied by the present kinetic treatment.

[8] Measurements of the influence of temperature on K_m and k', analogous to those previously reported for other substrates for chymotrypsin (5), will be reported later, as well as similar studies on carboxypeptidase and trypsin substrates.

entropy of activation will be greater, and the orientation of the substrate accordingly better, if this bond is present than if it is absent. On the basis of this line of reasoning, the secondary peptide bond contributes both to the affinity of the substrate for the enzyme and to the catalytic effect.[9] Comparison of the rates of activation of the substrates listed in Table I agrees, at least qualitatively, with this interpretation: Introduction of a chlorine atom on the α carbon of MHC, as in MCPP, increases the activation rate 10-fold. This increase cannot be ascribed to a primary effect of the chlorine atom on combination with the enzyme, since both the enzyme-substrate dissociation constant, K_m, and the rate constant, k', are the same for both enantiomorphs of MCPP. The same increase in k' is achieved by introduction of a hydroxyl group so as to yield *d*-MPL, in which this group probably has the same steric relation to the enzyme as has the peptide group in D-BPME. In all these compounds, enzymatic hydrolysis occurs in the absence of a point of attachment otherwise furnished by the secondary peptide bond. If the hydroxyl group of MPL is in the *l*-position, however, a 6-fold increase in the rate of activation over that of the *d*-isomer occurs. This difference in activation rate cannot be ascribed to the size or electronegativity of the hydroxyl group, since the activation rates of the two optical isomers of MCPP, which likewise contain an electronegative substituent of approximately the same size as the hydroxyl group, are the same. Rather, the increased activation rate of *l*-MPL, as compared to the *d*-isomer, may be related to the influence of the hydroxyl group in the *l*-position on attachment to the enzyme surface, and may be compared to the considerably larger increase produced by the secondary peptide bond of L-BPME.

The problem of relating the nature of the residues, R, to the affinities of the resulting compounds for the enzyme is complicated by the failure of the present kinetic treatment of the data to separate k' from K_m.[4] However, certain qualitative deductions can be made from comparison of the values given in Table I. For example, it is evident that the affinity of *d*-MPL must be less than that of *l*-MPL since the K_m values of these compounds are in inverse order of their respective k' values (*vide supra*). By the same reasoning, the affinity of L-BPME must be greater than that of *l*-MPL. Since *dl*-MCPP has a higher K_m value than *d*-MPL, although their respective *k′* values are identical, it follows that the affinity of *d*-MPL for the enzyme must be less than that of *dl*-MCPP. According to this reasoning, the present substrates may be arranged in order of decreasing affinities for chymotrypsin as follows:

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[9] The contribution of the secondary peptide bond to catalysis must be such as to compensate for the increased loss of entropy.

L-BPME > *l*-MPL > *dl*-MCCP > *d*-MPL.

The place of MHC in this series is difficult to evaluate, its most probable position being below *l*-MPL and above *d*-MPL. The large difference in relative affinities of L-BPME and MHC is also evidenced by experiments on the enzymatic hydrolysis of a mixture containing both substrates. The observed rate of hydrolysis was due to L-BPME only, indicating that the relative affinity of MHC for the enzyme was too low to cause any measurable competitive inhibition.

The difference between the affinities of the optical isomers of each substrate decreases with decreasing affinity of the *l*-substrate for the enzyme. Thus, the affinities of the two optical isomers of BPME for the enzyme are so much different that, in a solution of the racemate, only the L-isomer is hydrolyzed, without any inhibitory effect of the D-isomer.[10] In a racemic solution of MPL preferential hydrolysis of the *l*-isomer occurs to completion, the *d*-isomer of lower affinity being subsequently hydrolyzed at a lower rate. In the case of MCPP both optical isomers have the same affinity for the enzyme and are simultaneously hydrolyzed at the same rate. These effects may be correlated with the characteristics of the postulated bonding between the residue, R, and the enzyme surface. Hydrogen bonding involving the secondary peptide bond (BPME) produces sufficient orientation of the substrate with respect to the enzyme to "freeze" the substrate in a fixed preferential configuration, which is favorable for activation when the substrate is in the L-form, and which cannot be attained when the substrate is in the D-form. If hydrogen bonding involves a hydroxyl group, as in MPL, orientation is less rigid and steric restrictions are less critical because of the smaller size of the residue, R. Hence, in the *d*-isomer, the more distant hydroxyl group will contribute to activation primarily because of its electronegative character. If the residue is a chlorine atom, as in MCPP, no hydrogen bonding by donation can occur, and hence, no preferential orientation of either of the two optical isomers is to be expected. The activation rates are precisely the same for both enantiomorphs, the contribution of the electronegative substituent being evident from comparison with the lower activation rate of the unsubstituted MHC.

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[10] Unpublished experiments.

ACKNOWLEDGMENTS

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SUMMARY

An attempt has been made in this investigation to evaluate the contribution of the secondary peptide bond of specific ester substrates for chymotrypsin to their susceptibility to enzymatic hydrolysis. Various structural analogs of a specific ester substrate were prepared and tested in which the secondary peptide bond was replaced by a hydroxyl, chlorine, or hydrogen residue.

Quantitative rate measurements have shown that the susceptibility of these compounds to chymotryptic hydrolysis decreased in the following order of substituents on the α carbon atom of methyl hydrocinnamate: benzamido $>$ hydroxy $>$ chloro. The same order applies to the antipodal specificities of these compounds. These findings have been correlated with the nature of the bond between these residues and the active groupings on the enzyme surface.

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A Manometric Actinometer for the Visible Spectrum

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The actinometer to be described in this paper is based upon the work of H. v. Tappeiner (1), who discovered the sensitization of photooxidation processes by dyes; upon that of S. E. Sheppard (2), who discovered the role of sulfur compounds, especially of allyl thiourea, in the photochemical process of photography; and upon the manometric and bolometric method developed at Dahlem for the measurement of the quantum yield of photosynthesis (3a,b). In 1926, Warburg and Gaffron (4) determined the quantum yield of photooxidation of allyl thiourea when sensitized by chlorophyll or porphyrin by these methods. The result was that 1 molecule of oxygen reacts, if 1 light-quantum is absorbed by the sensitizers, that means that the quantum yield was

$$\varphi = \frac{O_2}{h \cdot \nu} = 1.$$

Due to many circumstances, the technical procedure of 1926 was too complicated to be used for practical actinometric purposes. We have simplified the procedure in such a way that quantum intensities can now be measured in the greater part of the visible spectrum by the chemical process of photooxidation wherever simple manometric equipment is available. We replaced the differential manometer by the simple Haldane-Barcroft manometer. Acetone, which because of its high vapor pressure, is unsuitable for manometric experiments, was replaced by pyridine, which, thus far, is the most appropriate solvent we have found. Allyl thiourea was replaced by unsubstituted thiourea, a simplification by which we eliminated a slow dark reaction.

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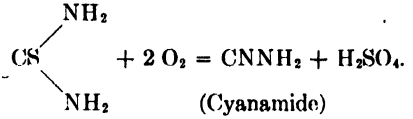
We have tried many substances as reductants in our system but none equaled thiourea. Even substances such as hydroquinone, which are easily oxidized by many oxidants, could not be photooxidized at all. Thus, Sheppard's discovery of the specific reactivity of certain sulfur compounds proved as important in photooxidation as in photography.

We have tried many dyestuffs but none equaled chlorophyll or prophyrin as sensitizers. We used crystalline ethyl chlorophyllid and crystalline protoporphyrin. As light absorbers they are complementary, the chlorophyll transmitting the green and the protoporphyrin transmitting the red. If mixed together they absorb the greater part of the visible spectrum.

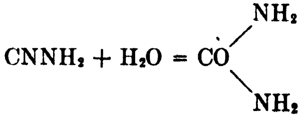
Photooxidation of Thiourea

One mg. of thiourea (13.2 micromoles) was dissolved in 5cc. of pyridine. One-tenth mg. of protoporphyrin was added. The mixture was introduced into a rectangular vessel of 14 cc. volume which was connected to a manometer. The gas space contained air. When shaken in the thermostat at 20°C. and illuminated with a 200 w. incandescent lamp at 20 cm., 135 mm.^{3} O_2 were absorbed during the first 10 min. When, after 230 min., the oxygen uptake had become very slow, 735 mm.^{3} O_2 had been absorbed, that is, 2.5 moles O_2/mole of thiourea. Only 2 moles of oxygen are calculated when the thiourea is oxidized according to the equation:



$$CS\begin{matrix}\diagup NH_2\\ \diagdown NH_2\end{matrix} + 2\,O_2 = \underset{\text{(Cyanamide)}}{CNNH_2} + H_2SO_4.$$

The pyridine solution was evaporated *in vacuo*, the residue taken up in water, and the insoluble porphyrin removed by filtration. In the filtrate, about 74% of the calculated amount of H_2SO_4 was precipitated by $BaCl_2$. To another portion of the solution $AgNO_3$ and ammonia, or copper acetate and ammonia, were added to test for cyanamide. Silver produced a dark yellow precipitate and copper a black precipitate. Another portion of the solution was tested for urea by urease, which was added from the sidearm of a manometer vessel to the acidified solution (pH 5.5). Seventeen per cent of the thiourea was recovered as urea. This urea may have been formed by hydrolysis of cyanamide:



$$CNNH_2 + H_2O = CO\begin{matrix}\diagup NH_2\\ \diagdown NH_2\end{matrix}$$

Finally, it may be mentioned that the protoporphyrin is slowly photooxidized in the solution. Spectroscopic examination showed the appearence of a band in the red at about 675 mμ. Very probably this is the first step in the transformation of blood pigment to bile pigment discovered in 1930 (5).

Actinometer

Two hundred mg. of thiourea, dissolved in 5 cc. pyridine ("medicinal" grade, Mallinckrodt), are introduced into a rectangular vessel of about 14 cc. volume. Two mg. of crystalline ethyl chlorophyllid are added, if red, yellow, or blue light is to be measured. Ten mg. of crystalline protoporphyrin are added if green or blue light is to be measured. The vessel is then connected to a simple manometer of the type used for respiration measurements. Pure oxygen is introduced into the gas-space. The vessel is shaken in the thermostat at 20°C. in the dark, until the pressure remains constant for at least 15 min. If the vessel then is illuminated by light which is absorbed by the chlorophyll or the porphyrin, thiourea is oxidized and the oxygen pressure in the vessel decreases.

It is an essential feature of this arrangement that the concentration of the chlorophyll or the porphyrin is so high that light entering in a vertical direction through the bottom of the vessel is completely absorbed, even when the vessel is in motion. To test this condition it must be shown that the oxygen consumption remains constant if the concentration of the dyes is increased.

Quantum Yield

The light beam leaving the slit of a monochromator was collimated by lenses and reflected by a mirror vertically into the vessel. The cross-section of the beam entering the vessel was about 2 cm.^{2}, whereas the bottom area of the vessel was about 9 cm^2. The light beam was adjusted to the middle of the vessel. Thus, when the vessel was shaken, no part of the beam passed outside the vessel.

The total intensity of the light beam was measured bolometrically as described elsewhere (5). The bolometer had been calibrated against a standard lamp of the U. S. Bureau of Standards. The quartz window, which protected the strips of the bolometer, was 2 mm. thick and transmitted 82.5% of the radiation of the standard lamp.

The actinometer was shaken in the ordinary shaking-machine (3a) with horizontal (not circular) motion. Foaming never occurred. When equilibrium was reached in the thermostat in the dark at 20°C., the pressure was noted. The manometer was then taken out of the thermostat and the shaking continued in air at about 20°C. Here the actinometer was illuminated with light of measured intensity. After periods of 10–30 min., the illumination was interrupted, the manometer was again placed in the thermostat and, after shaking 5 min. in the dark, the pressure was read.

The purpose of this procedure was to avoid uncertain corrections for the light transmission of the windows of the thermostat and of the water in the thermostat. The same mirror which reflected the light beam in a vertical direction into the actinometer, by rotation could reflect the same light beam into the bolometer. If I was the total intensity of the beam which reached the strips of the bolometer, $1.04 \cdot I$ was the total intensity which entered the actinometer. Thus, the corrections for the different paths of the light were reduced to a minimum.

We have tried to determine whether shaking is necessary during illumination, and always found distinctly smaller quantum yields when resting vessels were illuminated. The layer of the solution in which the main part of the light is absorbed is very thin and it seems that here, in the resting vessels, concentrations decrease to such a degree that light energy is lost.

Here follows an example of determination of the quantum yield:

$$20°\text{C.};\ 100\%\ O_2;\ 585\text{–}615\ \text{m}\mu\ (600\ \text{m}\mu).$$

$$V = 14.10\ \text{cm.}^3\ v_F = 5\ \text{cc.}\quad v_G = 9.10\ \text{cc.}\ K_{O_2} = 0.893.$$

200 mg. thiourea. 2 mg. ethyl-chlorophyllid, dissolved in pyridine. The solubility of O_2 at 20°C. in the actinometer solution was determined manometrically. $\alpha = 0.092$ was found and used for the calculation of the vessel-constant $K_{O_2} = \dfrac{v_G \frac{273}{T} + v_F \cdot \alpha}{10}$ [mm.²]. The total light intensisy entering the vessel was $I = 0.109$ micromoles of quanta/min. The manometer readings were:

20 min. dark...........	0
15 min. light 5 min. dark	−39 mm.

$$= -34.8\ \text{mm.}^3 = \frac{34.8}{22.4} = 1.5\ \text{micromoles}\ O_2.$$

$$\varphi = \frac{1.55}{15 \times 0.109} = \frac{1.55}{1.64} = 0.945.$$

In the following table are presented the results of a continuous series of experiments, the time of illumination being 30 min. in the blue or 15 min. in the other spectral regions. In each of the four spectral regions mentioned in the table, the light was tested for unabsorbed light, especially for infrared. Between the exit slit of the monochromator and the bolometer we placed an absorption cell of 1 cm. thickness. When the cell contained acetone or pyridine, it transmitted 91% of the light, the loss being effected only by reflection. When 0.2 mg.

of ethyl-chlorophyllid per cc. of acetone were added, the transmission in the blue, the yellow or the red was nearly zero; when 1 mg. of protoporphyrin per cc. of the pyridine was added, the transmission in the green was nearly zero.

It follows, from the data recorded in the table, that the accuracy of the method is sufficient for most photochemical experiments, inorganic or biological. The light intensities mentioned in the table should not be exceeded. One micromole of quanta entering the vessel in a period of 15 min., is about the right intensity. The pressure changes then are about 20 mm. and can be read with an accuracy of 5%.

Wavelength (mμ)	Light absorbed (micromoles quanta)	Oxygen consumed (micromoles O_2)	$\varphi = \frac{\text{micromoles } O_2}{\text{micromoles quanta}}$
640–660 (red)	1.98	1.82	0.92
640–660 (red)	0.99	1.02	1.03
505–525 (green)	1.14	1.10	0.96
420–450 (blue)	0.36	0.42	1.16
585–615 (yellow)	1.90	1.79	0.94
585–615 (yellow)	0.95	0.96	1.01
420–450 (blue)	0.44	0.46	1.04
640–660 (red)	1.65	1.47	0.89
640–660 (red)	0.825	0.78	0.94
640–660 (red)	0.83	0.83	1.00
585–615 (yellow)	1.64	1.55	0.95
505–525 (green)	0.88	0.84	0.95
			Average = 0.98

We started our experiments with dioxane as solvent. If it was pure and free of peroxide, the quantum yield seemed to be one. Otherwise, the yield was greater and could be decreased by cyanide or increased by copper salts. When peroxide-free dioxane, was kept overnight in a bottle which was not airtight it gave a strong peroxide reaction with KI. Therefore, dioxane was discarded as a solvent. Thus far we have not found a more satisfactory solvent than pyridine, which has a sufficiently low vapor pressure and dissolves thiourea, ethyl chlorophyllid and protoporphyrin sufficiently.

Applications

The actinometer seems particularly suitable for determinations of the quantum yield in photosynthesis. We first place the actinometer in the path of a light beam whose intensity is unknown and measure the oxygen uptake. In the same position we then place a vessel of the same dimensions filled with living green cells which also absorb the light completely. If we compare equal periods of irradiation, we obtain the quantum yield of photosynthesis by the simple equation:

$$\varphi = \frac{O_2 \text{ developed}}{O_2 \text{ absorbed}} = \frac{O_2}{h\nu}.$$

The general usefulness of the actinometer becomes apparent if we bear in mind that the instrument integrates the light intensities over time and number of quanta. If, for example, the vessel is shaken in light whose intensity and spectral distribution change with time, then one single manometric reading taken at the end of a given period gives the number of quanta which have entered the vessel during the whole period.

There is one limitation of the instrument: *i.e.*, that the light entering the vessel must be completely absorbed. Under the conditions given above, the instrument can be used from 670 mμ in the red to the blue end of the visible spectrum. How far it can be used in the ultraviolet must still be investigated. Quartz vessels must be used instead of glass vessels.

Direct Photooxidation of Thiourea

Two hundred mg of thiourea, dissolved in 5 cc. pyridine, were introduced into the rectangular glass vessel and illuminated—without addition of chlorophyll or porphyrin—by a 100 w. mercury vapor lamp in a "black glass" envelope, at about 15 cm. distance. In 45 min., 24 mm.^{3} O_2 were taken up. This experiment shows that thiourea, if it absorbs light, reacts with moelcular oxygen, and seems to support the theory of v. Tappeiner that the sensitizing dyes transfer energy, not oxygen, to the reductant.

SUMMARY

A chemical actinometer has been described, by which quantum intensities in the greater part of the visible spectrum can be measured manometrically.

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Concentration of DDT in the Blood and Tissues of Sheep Fed Varying Levels of DDT [1,2]

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INTRODUCTION

Lieberman (8), Smith and Michelbacher (13), and Lieberman and Hare (9), have shown that DDT effectively controls lygus bugs (*Lygus elisus* Van D., *Lygus hesperus* Knight) and alfalfa weevil (*Hypera postica* Hon.) in the production of seed alfalfa. The widespread use of DDT for controlling insects, especially alfalfa weevil, in the production of alfalfa hay further indicates the versatility of this insecticide. However, prior to its general acceptance as an insecticide for the control of insects in forage alfalfa production, it should be demonstrated that the residual DDT on the alfalfa hay is not harmful to domestic animals. These considerations prompted a series of studies in our laboratories on various domestic animals to gain information on this important problem.

Numerous papers have been published on the toxicity of DDT and on its accumulation in animal tissues. Leary *et al.* (7) give an excellent summary of the work done prior to 1946 in their book, "DDT and the Insect Problem." Orr and Mott (10) administered orally comparatively large doses of DDT to cows, horses, and sheep and the animals exhibited severe toxic symptoms. *Post mortem* examination demonstrated the accumulation of DDT in the tissues of the animals in relatively large amounts, particularly in the fat. Wilson *et al.* (15) fed DDT-treated pea vine silage (1 lb. DDT/ton of silage) to dairy cows and sheep. Although DDT accumulated in small amounts in the tissues of these animals and was present in the milk of the dairy cows in small amounts, the animals were apparently in good health. These workers concluded that DDT as used in their experiment presented a negligible hazard to human health. Carter (1) and Fitzhugh (4) summarized the results of experiments on domestic and laboratory animals in which DDT was found to have accumulated in small amounts in the tissues. Lardy (6) reported no apparent harmful effects following the feeding of DDT-treated corn silage to steers and milk cows. Carter *et al.* (2) claimed

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[2] Part of this data was presented at the Agriculture and Food Division of the Portland meetings of the Am. Chem. Soc., Sept. 16, 1948.

that the cooking of meat from DDT-treated animals did not change its DDT content appreciably, and (3) that DDT accumulated in the fat of pigs fed beef that contained DDT.

The purpose of the present work was to analyze the blood and tissues of sheep for DDT, following a period of feeding with alfalfa hay that had been dusted with DDT in approximately the same amounts used by hay producers. DDT was also fed in capsules at approximately the same level as that present on the alfalfa hay, and at higher levels, in order to determine a possible margin of safety for feeding DDT-treated alfalfa hay.

DESIGN OF THE EXPERIMENT

The alfalfa hay used in feeding the sheep was obtained from a 44 acre field near the College. This field was divided into 16 one-acre plots. Each plot was separated from its neighbor by a buffer strip which served to protect it from drift of DDT during the application of the insecticide to the alfalfa. Four plots in each of 4 blocks of plots were assigned randomly to each of the following treatments: (1) no DDT, (2) 1 lb. of technical DDT/acre, (3) 2 lb. of technical DDT/acre, and (4) 4 lb. of techincal DDT/acre. This gave four replications of each of the four treatments. One to 2 lb. of technical DDT/acre is the amount usually used in this area for controlling insects in alfalfa hay production.

The DDT in a pyrophyllite carrier (as a 5% DDT dust) was applied to the alfalfa with a power duster. The time of application was determined by making counts of the insect population on each of the plots. Insect counts were also made periodically between the time of application of the DDT and harvest of the hay to determine the degree of control obtained. These data will be published elsewhere.

The harvested hay was analyzed for DDT, protein, ether extract, crude fiber, ash, nitrogen-free extract, phosphorus, calcium, and carotene. The detailed data will be published elsewhere (5).

A total of 64 wether lambs, 32 Rambouillett, and 32 Columbia × Rambouillet were used in the experiment. They were approximately 5 months of age and weighed 60–80 lb. at the beginning of the experiment.

Thirty-two lambs, 16 Rambouillet, and 16 of the crossbred Columbia × Rambouillet were selected at random from the original 64 lambs. Four lambs of each breed were then assigned to receive hay from each of the 4 replications mentioned above, giving a total of 8 lambs on each of the 4 alfalfa treatments.

The remaining 32 lambs were segregated in the same manner as the above group. These lambs were fed similar, but untreated, alfalfa hay. However, in addition, they received an accurately weighed amount of technical DDT in capsules which were force-fed daily. The 4 groups of lambs received the following treatments: (1) no DDT, (2) 50 parts DDT/million of hay eaten, (3) 100 p.p.m. DDT, and (4) 200 p.p.m. DDT. The quantity of DDT given was calculated on the basis of the amount of hay eaten by the individual lamb during the previous two week period. Four lambs of each breed were assigned to each of the 4 treatments as in the previous group, making a total of 8 lambs on each treatment.

All of the lambs were fed in individual pens. The diet of alfalfa hay was supplemented with a feed mixture consisting of beet pulp, barley, salt, and bone meal. Records of food intake were kept, and the lambs were weighed every 2 weeks. The lambs were fed approximately 50% alfalfa hay and 50 % of the feed mixture.

At the end of a 4 month feeding period, a sample of venous blood was obtained from each animal for the determination of calcium, phosphorus, hemoglobin, and DDT. The animals were then killed and samples of mesenteric and kidney fat (these were combined into a single sample which was analyzed), muscle, liver, kidney, and brain were taken for DDT analysis. Tissue samples were also obtained for histological study. The data on weight gains, calcium, phosphorus, and hemoglobin content of blood, and the histological study will be published elsewhere (5).

METHODS OF ANALYSIS

After preliminary studies for the determination of DDT, the total chloride method of Umhoefer (14) and the specific colorimetric method of Schechter *et al.* (12) were selected. Good recovery of added DDT (95–98%) was obtained by these methods in our laboratory.

Determination of the DDT content of the hay from each plot was made by the two methods selected (12,14). The data obtained by both methods were in good agreement. In the analysis of the hay samples by the Umhoefer method (14), the benzene extract from the samples was concentrated by vacuum distillation, refluxed with sodium in isopropanol, and titrated according to Volhard using standard $AgNO_3$.

Analysis of all the tissues and the blood for DDT was made by the method of Schechter *et al.* (11,12), with the following modifications: (1) a 5 g. fat sample was mixed in a Waring blendor with 50 ml. of chloroform and the extract was transferred directly to the 50 ml. separatory funnels, (2) the remainder of the tissues (40 g. muscle tissue, 50 g. liver, 50 g. kidney, and 75 g. brain) was mixed with 50 ml. of water and 50 ml. of ethanol in a Waring blendor after which the mixture was placed in 250 ml. centrifuge bottles with Skellysolve B. The addition of the water-ethanol mixture served to precipitate the protein and to prevent the formation of emulsions with the Skellysolve. The DDT was extracted from the tissues with the Skellysolve B, which, after centrifuging, was easily separated from the remainder of the mixture. From this point the analysis was carried out as with milk in the method of Schechter *et al.* (11,12).

All colorimetric readings were made on the Beckman quartz spectrophotometer.

RESULTS AND DISCUSSION

None of the lambs showed ill effects from the ingestion of DDT. Two lambs died from injuries which were not associated with DDT feeding. The remainder were in apparent good health during the entire experiment. There was no significant difference in live-weight gain between any of the lambs on any of the DDT treatments or between any of the DDT levels and the control lambs (5).

TABLE I

DDT Content of Tissues of Lambs Consuming DDT-Treated Alfalfa Hay[a]

DDT applied/acre	DDT residue on hay	DDT in mesenteric and kidney fat	DDT in muscle	DDT in liver	DDT in kidney	DDT in brain
lb	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m*	*p.p.m*
0	0	1.9±1.1	0.0	0.0	0.0	0.0
1	15	15.8±8.0	0.4±0.3	1.3±0.3	>0.1	0.1
2	22	18.2±8.0	0.6±0.4	1.3±0.5	>0.1	>0.1
4	42	44.2±18.3	0.9±0.3	1.5±0.6	0.18±.09	0.2±.05

[a] Each value is the average ± S. D. of 8 lambs.

The amount of DDT present on the DDT-dusted hay, and in the mesenteric and kidney fat, muscle, liver, kidney, and brain of the lambs consuming it, is shown in Table I. It will be noted that the DDT residue on the alfalfa hay varied from 0 to 42 p.p.m., the undusted alfalfa containing no DDT and the dusted alfalfa containing amounts that increased progressively with the quantity applied. After the lambs consumed the alfalfa hay for a period of 4 months, the mesenteric and kidney fat contained a substantial amount of DDT, which again varied with the quantity present on the hay that was eaten. The presence of a small amount of DDT in the fat of the lambs receiving untreated hay might be explained on the basis of chance consumption of treated hay during the daily feeding activities. The possibility of this small amount of DDT being due to technical error would seem to be negated by the absence of any DDT in the remaining tissues of the control sheep (Tables I and II). The presence of DDT in trace amounts, however, does not detract from the significance of the other data. It is interesting

TABLE II

DDT Content of Tissues of Lambs Receiving DDT in Capsules[a]

DDT fed in capsules	DDT in mesenteric and kidney fat	DDT in muscle	DDT in liver	DDT in kidney	DDT in brain
p.p.m. of hay	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
0	0.0	0.0	0.0	0.0	0.0
50	19.6±6.2	0.6±0.2	0.5±0.3	0.10±.07	0.15±.06
100	32.1±5.8	0.6±0.3	0.7±0.2	0.09±.05	0.18±.05
200	73.4±29.2	1.6±1.1	1.7±1.1	0.41±.5	0.30±.18

[a] Each value is the average ± S. D. of 8 lambs.

that the amount of DDT present in mesenteric and kidney fat closely parallels the amount present on the hay.

The DDT content of mesenteric and kidney fat, muscle, liver, kidney and brain of lambs fed DDT in capsules is presented in Table II. As before, the greatest quantity of DDT was present in mesenteric and kidney fat, and only traces were found in the other tissues. The amount of DDT present in the fat was roughly proportional to the amount ingested. In these lambs, however, the same amount of DDT was not present in the fat as was fed in capsules. This variation from the data in Table I probably indicates that in capsule feeding there is less net retention of DDT than when treated hay is fed. In lambs consuming DDT-treated hay, it would seem that the ingestion of smaller amounts of DDT over a much longer daily period, owing to the more or less intermittent eating routine, would maintain the blood DDT level lower and more constant, thereby allowing a more complete concentration of the DDT in the fatty tissues. Additional studies are needed, however, to establish this hypothesis.

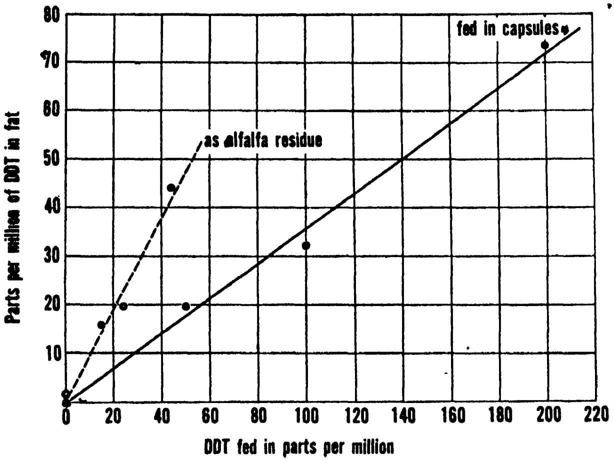

FIG. 1. Relationship between DDT ingested and its concentration in mesenteric and kidney fat.

Only trace amounts of DDT were found in the muscle, liver, kidney, and brain.

Analysis of the blood showed no DDT present in the blood of any of the lambs at the time of slaughter. In making the analyses, 100 ml. of venous blood were used; consequently the results would seem to indicate quite clearly that only very minute amounts of DDT, or no DDT were present in the blood at the time the sample was drawn.

The relationship between DDT ingested and its concentration in the mesenteric and kidney fat is shown in Fig. 1. It will be noted that, in both capsule feeding and feeding as a residue on the alfalfa, approximately a straight-line relationship exists between the quantity of DDT ingested and the quantity stored in the fat.

Investigations similar to those reported here were made by Wilson *et al.* on sheep (15), and Carter *et al.* on pigs (3). Wilson *et al.* fed DDT-treated pea vine silage to 30 lambs and 20 pregnant and nursing ewes. The lambs received an estimated 62 p.p.m. of DDT in their diet, while the ewes received an estimated 106 p.p.m. *Postmortem* analysis of the tissues of 2 of the lambs and 4 of the ewes showed the presence of 5 p.p.m. of DDT in the muscle and 96 p.p.m. in the fat of the lambs, while the muscle of the ewes contained 1 p.p.m. of DDT and the fat 62 p.p.m.

In the investigations of Carter, beef containing DDT was fed to two lots of 6 pigs each. The beef was obtained from the carcasses of steers which had eaten hay containing residual DDT. Lot 1, which was fed a diet containing 5 p.p.m. of DDT, had stored 5 p.p.m. of DDT in the lean meat and 16–18 p.p.m. in the fat. Lot 2, which was fed a diet containing 2 p.p.m. of DDT, was found to contain 1.7 p.p.m. of DDT in the lean meat and 11–15 p.p.m. in the fat.

The results of this study indicate that considerable quantities of DDT may accumulate in the fatty tissues following the regular ingestion of relatively small amounts of DDT, and that it is present in only small amounts in the other tissues. Even though the daily intake was not sufficient to produce toxic symptoms during the 4 month feeding period the possibility exists that sufficient DDT may accumulate in the fat if its ingestion is continued over a long period of time, so that eventually harmful effects may be produced in the animal or in humans consuming the meat. This aspect of the problem requires further study, however.

SUMMARY

Alfalfa hay that was dusted with DDT for insect control was fed to fattening lambs. The amount of DDT used was 1, 2, and 4 lbs of technical DDT per acre in a pyrophyllite carrier. Another group of lambs

received DDT in capsules by mouth, the amount being 50, 100, and 200 p.p.m. of alfalfa hay consumed. The feeding period was approximately 4 months. Eight lambs were in each group receiving dusted hay and DDT in capsules, and there were 16 control lambs giving a total of 64 lambs in this study.

The average DDT residue on the dusted hay was 0, 15, 22, and 42 p.p.m. when 0, 1, 2, and 4 lb. of technical DDT per acre, respectively, were applied.

No DDT could be detected in the blood of any lambs, at the time of slaughter.

The highest concentration of DDT was found in the mesenteric and kidney fat. The average amount of DDT present was 1.9, 15.8, 18.2, and 44.2 p.p.m. in the lambs consuming alfalfa hay dusted with 0. 1, 2, and 4 lb. of technical DDT per acre, respectively. The average amount present in the mesenteric and kidney fat of lambs receiving DDT in capsules was 0, 19.6, 32.1, and 73.4 p.p.m. when 0, 50, 100, and 200 p.p.m. DDT, respectively, were fed.

The average amount of DDT found in muscle, liver, kidney, and brain varied from 0 to 1.7 p.p.m.

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Structure of the Pigment of Macpalxochitl

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INTRODUCTION

The "*flor de manita*" or "macpalxochitl" (macpal = hand, xochitl = flower) is a single, angular, hermaphroditic flower. Its calyx measures about 3 cm. across and is divided into 5 parts. The outer surface has a reddish coffee color, and the inside an intense red color. There are 5 stamens, red in color, united at the base to form a column, and giving the appearance of a hand or the claw of a bird (1).

The object of the present work is the isolation of the red pigment (a glycoside) of the "*flor de manita*" and the determination of the structural formula.

EXPERIMENTAL

1. Preparation of the Material

About 1000 blossoms were exposed to the sun to dry for 4 days. When dry they were pulverized, the total weight at this stage being 1 kg. The powder was defatted by extractions with gasoline, repeated until a drop of the solvent placed on filter paper left no residue. Two final extractions were carried out with petroleum ether and the residue allowed to dry. The defatted dry powder weighed 950 g.

2. Preparation of the Glycoside

A crude picrate of the glycoside was prepared by the method of Anderson (2). This was digested with 90 cc. of methyl alcohol containing 15 cc. of 20% HCl in methyl alcohol. Filtration yielded an intensely red colored solution from which the pigment was precipitated by addition of 600 cc. of anhydrous ether. The red, amorphous picrate was collected on a Büchner funnel, washed with ether, and dried. Yield: 960 mg. from 950 g. of dried fat-free material. To remove the last trace of picric acid, the amorphous material was moistened, and suspended in a medium of 30 cc. anhydrous ether containing 1 cc. of 20% HCl in methanol. After stirring this mixture for 2 hr.

the picrate was filtered off and washed with ether until the washings left no residue on evaporation. After drying in a vacuum desiccator the solid weighed 930 mg.

Anal. for $C_{54}H_{53}O_{31}Cl$. Calc.: C, 52.5; H, 4.3; Cl, 2.8. Found: C, 52.8; H, 4.4; Cl, 3.1.

Molecular weight for $C_{54}H_{53}O_{31}Cl$ (cryoscopic method). Calc. 1232.5; found 1235.

3. *Preparation of the Aglycone*

The glycoside (900 mg.) was dissolved in 15 cc. of water to which was added 15 cc. of concentrated HCl, and the solution was then boiled under a reflux condenser for 5 min. After boiling for about 1 min., prismatic crystals of the chloride began to form. The mixture was placed in a refrigerator over night. The crystals obtained were washed with HCl and dried in a vacuum desiccator containing H_2SO_4 and KOH. The product consisted of uniform homogeneous crystals with a total weight of 460 mg. and, after drying at 105°C., was subjected to molecular analysis.

Anal. for $C_{15}H_{11}O_4Cl$. Calc.: C, 61.9; H, 3.7; Cl, 12.2. Found: C, 62.1; H, 3.9; Cl, 12.6.

Molecular weight for $C_{15}H_{11}O_4Cl$ (Rast method). Calc. 290.5; found 295.

Preparation of the anthocyanidin chloride was repeated 4 times to yield sufficient material for subsequent experiments.

4. *Isolation of Glucose as Phenylglucosazone*

The phenylglucosazone was obtained from the filtrate of the anthocyanidin chloride again following the technique of Anderson (3). A heavy yellow precipitate was obtained which melted with decomposition at 208°C. For corroboration the *p*-nitrophenylhydrazone and acetate were prepared; the *p*-nitrophenylhydrazone melted at 189°C., and the acetate at 112°C., both corresponding to the values listed in the literature for D-glucose (4).

Quantitative determination of glucose by the spectrophotocolorimetric method demonstrated the presence of 3 molecules for each molecule of aglycone.

5. *Preparation of the Acetylated Derivative of the Anthocyanidin*

Six hundred mg. of the anthocyanidin chloride was acetylated by the method of Einhorn and Hollandt (5). The acetylated derivative was separated as a fine amorphous precipitate of a light red color. The purified product weighed 740 mg. It was readily soluble in pyridine and in acetone, producing a coffee-colored solution. It was moderately soluble in hot ethanol or methanol, but insoluble in water or dilute acid, although easily soluble in dilute alkali with the development of a green color showing a red cast in the light. On heating the alkaline solution, the green color was replaced by red. On heating with dilute acid, the color passed to a brilliant red, probably as the result of hydrolysis. The coffee-colored solution in alcohol was also transformed to red by the addition of HCl and heat.

The acetylated derivative contained 3 acetyl groups, as determined by hydrolysis with 30% H_3PO_4 and titration of the acetic acid liberated by distillation.

Anal. for $C_{21}H_{17}O_7Cl$. Calc.: C, 60.5; H, 4.1; Cl, 8.5. Found: C, 60.8; H, 4.4; Cl, 8.9.

Anal. for 3 CH_3CO. (cc. of 0.1 *N* KOH); Calc., 25.84; found, 26.21.

6. *Oxidation of the Acetylated Derivative by Neutral Permanganate*

The acetylated product (0.6 g.) was suspended in 80 cc. of water containing 1.5 g. of $MgSO_4$. The mixture was kept at room temperature and stirred constantly during the slow addition of 2.6 g. of powdered $KMnO_4$ over a period of 3 hr. After standing over night, the solution was colorless. After acidification with H_2SO_4, a current of SO_2 was passed through the mixture until all the MnO_2 was dissolved. The filtrate containing the oxidation products was extracted with 5 portions of ether, and the ether removed by distillation. An acidic residue remained which solidified on the addition of water. This substance was dissolved by the addition of $NaHCO_3$. The solution was filtered, and diluted to 20 cc. with water acidified with HCl. When a faint turbidity appeared and a small quantity of resinous material separated, the solution was heated to 50°C. and shaken with Norit. It was then filtered and concentrated to 4 cc. by vacuum distillation. During the concentration, and after cooling the solution, crystals began to form. These were filtered off, washed with water, and dried in a vacuum desiccator. Further concentration of the mother liquor yielded a small additional quantity of crystals which were recovered and added to the first batch. The crystals weighed 0.07 g. and melted at 192°C., corresponding to *p*-acetoxybenzoic acid (6).

7. *Saponification of the p-Acetoxybenzoic Acid*

A quantity of 0.1 g. of the crystals was refluxed for 1.5 hr. in 10 cc. of water and 3 cc. of H_3PO_4; the needles which separated on cooling were filtered and washed with water. The crystals were dissolved in hot water, treated with Norit, and the filtrate again allowed to crystallize. Colorless crystals formed which melted at 213°C., corresponding to the melting point of *p*-hydroxybenzoic acid (7). For confirmation, the S-benzylthiuronium-*p*-hydroxybenzoate was prepared, following the technique of Veibel and Ottung (8). The substance obtained melted at 145°C., conforming with the value given in the literature.

8. *Preparation of Phloroglucinol By Alkaline Fusion of the Anthocyanidine*

Two hundred mg. of anthocyanidine chloride were fused with KOH according to the technique of Boehm (9). After extraction with ether and evaporation of the solvent the product obtained was dissolved in pyridine. On evaporation of the pyridine, crystals formed which, after recrystallization, melted at 219°C., corresponding to the melting point of phloroglucinol. For confirmation, the tribenzoate was prepared according to the method of Skraup (10); the product melted at 173°C., as described for 1,3,5-tribenzoxybenzoate (phloroglucinol tribenzoate).

9. *Preparation of Gallic Acid*

From the mother liquor remaining after obtaining glucose by the method of Anderson, gallic acid was prepared according to the technique of Fischer (11). Purification was accomplished by means of the procedure of Feist (12). In this manner was obtained a substance melting at 253°C., the melting point described for gallic acid.

For confirmation, 3,4,5-triacetoxybenzoic acid was prepared, using acetic anhydride and small quantities of pyridine, following the technique of Fischer (13). The melting point of the product was 171°C., corresponding to that described in the literature.

DISCUSSION

The study of the colored glycoside of the "*flor de manita*" was made on a preparation of dried, powdered, defatted flowers from which the crude glycoside was extracted as the picrate by the method of Anderson. The picrate was converted to the chloride, giving a substance composed of carbon, hydrogen, nitrogen, and chlorine in the following proportions: C, 52.8; H, 4.4; Cl, 3.1. According to these results the empirical formula must be $C_{54}H_{53}O_{31}Cl$. Cryoscopic determination of the molecular weight confirmed this formula. The calculated molecular weight was 1232:5, and the weight determined was 1235.

Hydrolysis of the picrate with HCl yielded a substance whose simplest empirical formula must be $C_{15}H_{11}O_4Cl$. This substance was easily acetylated by the method of Einhorn and Hollandt.

Hydrolysis of the acetylated product with orthophosphoric acid revealed the presence of 3 hydroxyl groups. The acetylated derivative has the following minimal formula $C_{21}H_{17}O_7Cl$.

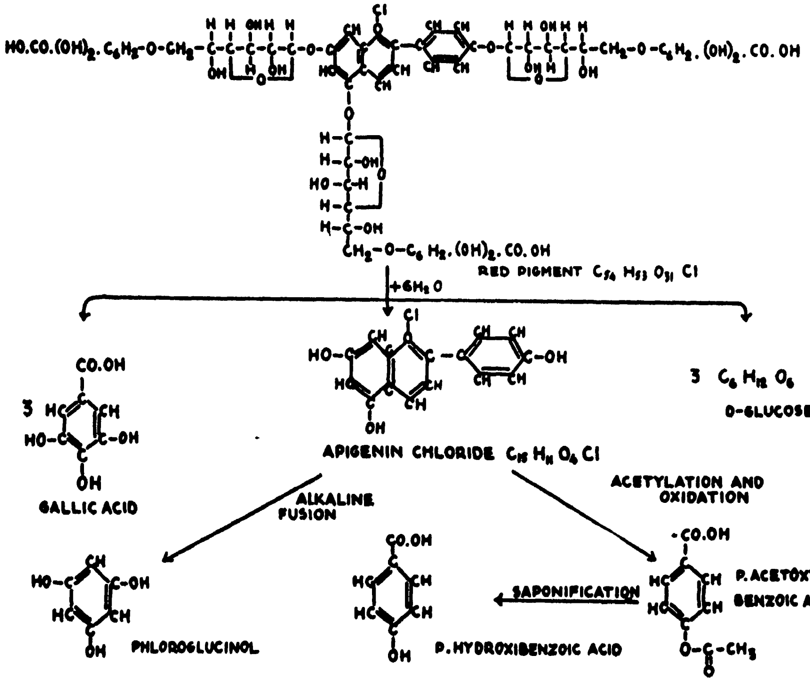
Oxidation of the acetylated derivative produced *p*-acetoxybenzoic acid which melted at 192°C. After hydrolysis with orthophosphoric acid, crystals were obtained of *p*-hydroxybenzoic acid melting at 213°C. For final confirmation the S-benzylthiuronium-*p*-hydroxybenzoate was prepared following the method of Veibel and Ottung. This product melted at 145°C., corresponding to the melting point described in the literature.

The substance $C_{15}H_{11}O_4Cl$ was subjected to alkaline fusion by the method of Boehm. Extraction with ether, and later with pyridine, yielded crystals melting at 219°C. (phloroglucinol). In confirmation, the tribenzoate was prepared, which melted as expected (173°C.).

From all the data given above we have concluded that the glycosidal pigment contained an anthocyanidine group which, in this case, was the chloride of apigenine.

From the products of hydrolysis, phenylglycosazone was isolated in the form of a heavy yellow precipitate, with a decomposition point of 208°C. To confirm the presence of glucose, the *p*-nitrophenylhydrazone and the acetate were prepared, melting at 189°C. and 112°C., respectively. By quantitative analysis it was determined that each molecule of anthocyanidine was attached to three molecules of glucose. From the mother liquors from which the glucose had been extracted, gallic acid was obtained (method of Fischer). Purified by Feist's method, this substance melted at 253°C., agreeing with the values in the literature. For confirmation, 3,4,5,-triacetoxybenzoic acid was prepared (Fischer); this substance melted at 171°C., again in agreement with the recorded datum.

The above data lead to the conclusion that the pigment of the "*flor de manita*" is composed of an aglycone, $C_{15}H_{11}O_4Cl$, plus three molecules of glucose and three of gallic acid. We offer the following structural formula consonant with the quantitative data cited above and the systematic degradation of the glycoside.



SUMMARY

A colored glycoside has been extracted from the "*flor de manita*," whose empirical formula is $C_{54}H_{53}O_{31}Cl$.

It has been demonstrated that the product is formed by the union of one molecule of apigenine chloride with 3 molecules of glucose and 3 of gallic acid. According to this, the pigment may be given the chemical name: Trigalloglucoxy-5, 7, 4′-chloro-1-benzopyroxone.

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Inhibition of Crystalline Lysozyme

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INTRODUCTION

Studies of the interaction of surface-active agents with enzymes have demonstrated that enzymes such as pepsin, urease, and catalase can be precipitated and completely inactivated when their electrical charge is opposite in sign to that of the surface-active agent (6,10). Furthermore, the activity of urease, trypsin and phosphatase can also be slightly inhibited by an anionic surface-active agent (7), while amylase, lipase and pepsin can be completely inhibited (5). The activity of crystalline trypsin can also be diminished or completely inhibited by interaction with soaps (9). On the other hand, anionic surface agents have been shown to activate certain enzymes. For example, protyrosinase can be converted to active tyrosinase by alkyl sulfates (2,4). When homologous series of alkyl sulfates were tested on protyrosinase, greatest activation was found when alkyl sulfates with carbon chains in the 12 to 16 carbon range were used.

Recently, it has been reported by Meyer *et al.* (8) that lysozyme can be inhibited by sodium dodecyl sulfate in concentrations as small as 0.0001 *M*. Similarly, it has been found by workers in this laboratory that sodium dodecyl sulfonate, sodium dodecanate, and dodecyl alcohol also inhibits crystalline lysozyme. However, when short chain alkyl sulfates were tested, no inhibiting action could be detected. This suggested a relationship between the length of the carbon chain and the inhibiting action of the compound. Therefore, experiments were conducted to determine whether the length of the carbon chain or a combined effect of chain length and functional group was the important factor in determining the inhibitor effect of the compounds in a homologous series.

This paper is an account of some of these experiments which have been conducted to ascertain the relationship between the structure of the compounds tested and their inhibitory action on crystalline lysozyme.

PROCEDURE

In a test tube (18 × 150 mm.) was placed from 0.1 to 1.0 ml. of the inhibitor solution together with sufficient water to give a final volume of 5 ml. An aliquot of 0.25 ml. of lysozyme solution was added and the solution allowed to stand at 37°C. After 15 min., 5 ml. of a suspension of *Micrococcus lysodeikticus* cells was added and the optical density of the mixture immediately determined at 550 mμ in a Coleman Junior Spectrophotometer. The solution was then incubated for 30 min. at 37°C. and the optical density again determined.

The decrease in the optical density of the solution was used as a measure of the extent to which the *micrococcus* cells had been lysed. A comparison between the control solution and the solutions containing the inhibitors was used to determine the inhibiting action of the compounds on crystalline lysozyme.

REAGENTS

Lysozyme Solution

Crystalline lysozyme prepared according to the method of Alderton and Fevold (1) was dissolved in 0.85% NaCl solution so as to have a final concentration of 1 mg. of enzyme/5 ml. of solution. Fresh solutions were prepared each day and stored at 3°C. when not in use.

Suspension of Micrococcus Cells

A suspension of *Micrococcus lysodeikticus* (American Type culture No. 4698) was prepared according to the method of Boasson (3). The suspension was diluted with 0.85% NaCl solution to an optical density of 1.0 as read in a Coleman Junior Spectrophotometer at 550 mμ. These suspensions remain stable for about 10 days when stored at 3°C.

Solutions of the Inhibitors

The soluble chain compounds were dissolved in water until a concentration of 0.005 *M* was obtained. In some cases, it was necessary to adjust the solution to pH 9 in order to completely dissolve the material. In such cases, the test aliquot was added to 3 ml. of distilled water, the pH adjusted to pH 7 and then the solution diluted to give the total volume of 5 ml. for the test.

The 0.005 *M* solutions of the water-insoluble compounds were prepared by dissolving the materials in ethanol. Aliquots of the alcoholic solutions were used directly in the test to determine the inhibitory action. In no case did the final concentration of alcohol in the test solutions exceed 10%. Similar concentrations of alcohol were added to the controls, even though it had been found that this small concentration of alcohol has no significant effect on the test.

RESULTS

The activity of crystalline lysozyme was found to diminish by interaction with alkyl sulfates. Fig. 1 shows that the degree of inactivation varies with the length of the carbon chain and the concentration of the sulfate used. When a homologous series of alkyl sulfates were tested, optimal inactivating efficiency on crystalline lysozyme was found in the 12 to 16 carbon atom range. Compounds containing 8 or less carbon atoms in the chain showed no inhibiting effect. The inactivating effect of the alkyl sulfates increased both with the length of the chain and with the concentration of the solution. In the carbon chain range of 12 to 16 atoms, the percentage of lysozyme inactivated increased approximately two times with each two carbon increment. Thus, tetradecyl sulfate (C_{14}) in concentrations of 1.0×10^{-4} *M* inhibited 38% of the lysozyme while the same concentration of dodecyl sulfate (C_{12}) inhibited only 18% of the enzyme. Similarly, hexadecyl sulfate (C_{16}) inactivated 88% of the enzyme, which is thus approximately twice as effective as tetradecyl sulfate and 4 times that of dodecyl sulfate.

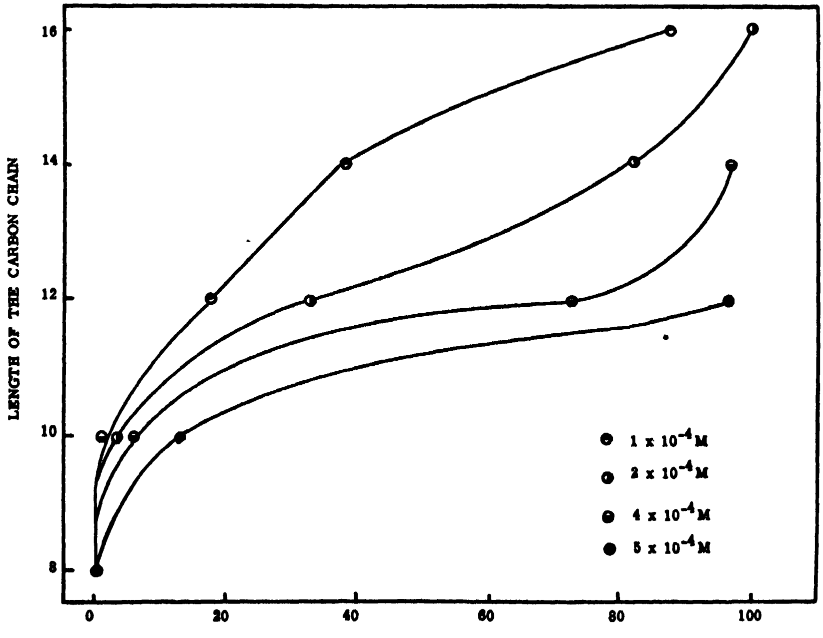

FIG. 1. Inhibition of crystalline lysozyme by alkyl sulfates.

The inactivating efficiency of the alkyl sulfates also increases with concentration. With decyl sulfate, slight inactivation of the lysozyme (5–12%) could be detected at concentrations of 4.0×10^{-4} to 5.0×10^{-4} *M*. A concentration of 5.0×10^{-4} *M* was needed to produce complete inhibition of crystalline lysozyme with dodecyl sulfate (C_{12}), while concentrations of 3.0×10^{-4} *M* and 2.0×10^{-4} *M* of tetradecyl sulfate (C_{14}) and hexadecyl sulfate (C_{16}), respectively, were sufficient to produce complete inactivation of the enzyme.

The straight chain fatty acids and their sodium salts also showed the same relationship between chain length and inhibiting action on crystalline lysozyme as was exhibited by the alkyl sulfates (see Fig. 2).

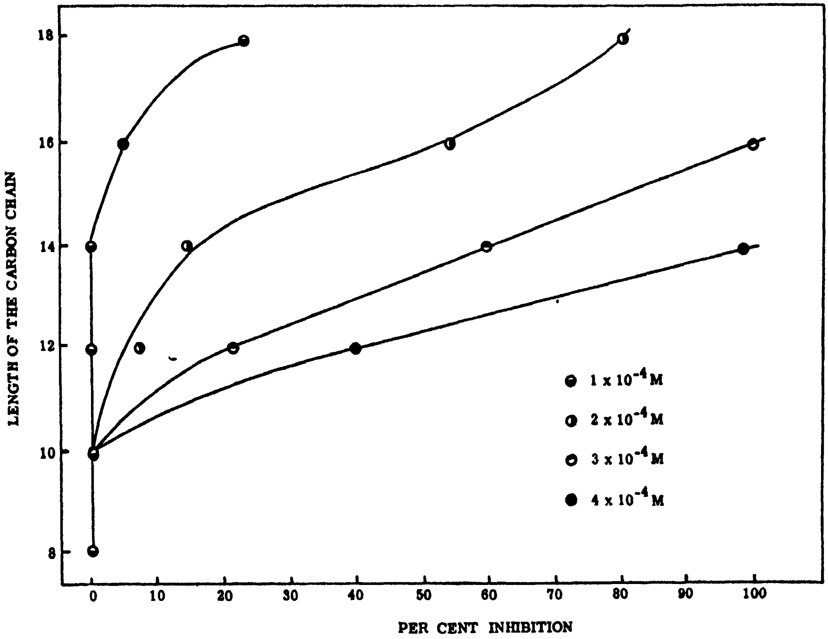

FIG. 2. Inhibition of crystalline lysozyme by the sodium salts of the long chain fatty acids.

The efficiency of the fatty acids and their sodium salts were, however, not as pronounced as was the case with the alkyl sulfates. The difference between the efficiency of the members of the fatty acid series and their corresponding counterpart in the alkyl sulfate series was probably due to the difference in solubility of the compounds. Although both series were tested using similar concentrations of the compounds, there will be considerable difference in the concentrations of the soluble components of each compound which will be present. Since it is the effect of the soluble component which is measured, there will be naturally a difference in the two series due to the differences in the solubility of the fatty acid series as compared with the alkyl sulfate series.

When a homologous series of the sodium salts of the long chain fatty acids were tested, inactivating efficiency increased in the range of 12 to 18 carbon atoms. Inactivation increased from 7% with dodecanate to 80% with octadecanate when a concentration of $2.0 \times 10^{-4}\ M$ was used. The increased inactivating effect in this series was not as regular as was the case with the alkyl sulfate series. The inhibition increases from 7 to 14, 54, and 80% with an increase in the carbon chain from 12 to 14, 16, and 18 carbon atoms, respectively.

The effect of any given fatty acid salt also increased with concentration. Sodium tetradecanate, for example, produced 0, 14, 60, and 98% inactivation with concentrations of 1.0×10^{-4}, 2.0×10^{-4}, 3.0×10^{-4}, and $4.0 \times 10^{-4}\ M$, respectively.

The action of the free fatty acids were in all cases less than those of their sodium salts. This variation was to be expected, due to the difference in solubility of the compounds as explained above.

A homologous series of straight chain alcohols were also tested. The inactivating effect of this series did not approach that of the other series. The compounds were, in general, insoluble in the test solutions and had to be introduced into the digests as alcoholic solutions. They produced only slight inhibiting effect, as compared with the other series tested (see Table I).

The results indicate that the inhibiting efficiency of a homologous series of straight chain alcohols also increases with chain length and concentration. The effect produced is, however, only slight, due to the small amount of the soluble component present in each test solution.

Results similar to those obtained with the homologous series of alkyl sulfates, fatty acids, sodium salts of fatty acids, and alcohols have been obtained with homologous series of mercaptans and acetates. The results in these cases are, however, not as conclusive as those from the other series. This is also due to marked insolubility of these compounds. In general, the more insoluble the compound, the smaller amount of its soluble component will be present in the test solution and other factors being equal, the less effect it will have on the enzyme.

TABLE I

Per Cent Inhibition of Crystalline Lysozyme with Various Straight Chain Alcohols

Chain length	Concentration				
	1×10^{-4} *M*	2×10^{-4} *M*	3×10^{-4} *M*	4×10^{-4} *M*	5×10^{-4} *M*
8	0	0	0	0	0
10	0	8	14	20	20
12	2	2	9	17	30
14	5	14	19	22	30
16	8	11	14	17	19
18	14	14	14	14	14

REVERSIBILITY OF ALKYL SULFATE INHIBITION OF LYSOZYME

Attempts were made to restore the activity of the lysozyme which had been inactivated by tetradecyl sulfate. Solutions containing lysozyme which had been partly inactivated with tetradecyl sulfate were dialyzed against dilute acetic acid solutions (pH 5), dilute NH_4OH solutions (pH 8), and distilled water (pH 7), at room temperature and 3°C. The activity of each enzyme solution was determined each hour for the first 6 hr. of dialysis. In no case was there any indication of regaining activity. During this period, the controls had shown no appreciable lose of activity.

The possibility of removing the alkyl sulfate inhibiting agent by precipitating it with barium or calcium ions was also tried. This procedure resulted in complete loss of activity. Apparently the barium and calcium ions form a complex with the alkyl sulfate which precipitates and removes the lysozyme from solution. The enzyme was probably removed either by absorption on the precipitate or by becoming a chamical part of the precipitate. In none of the cases tried was it possible to reactivate the lysozyme which had been inactivated by the tetradecyl sulfate.

THE EFFECT OF ALKYL SULFATES ON THE SPECIFIC CONDUCTIVITY OF LYSOZYME SOLUTIONS

Experiments were conducted to determine whether the differences in the inhibitory action of octyl sulfate and tetradecyl sulfate toward lysozyme could be explained on the basis of a difference in the combining capacity of these compounds with the enzyme. A comparison was made between the increased conductivity when 0.025 *M* solution of the alkyl sulfate was added in increments of 0.5 ml. to a solution containing 5 ml. of 0.85% NaCl and 95 ml. of conductivity water, and when it was added to a solution containing 20 mg. of crystalline lysozyme, 5 ml. of 0.85% NaCl solution and 95 ml. of conductivity water. The solutions were allowed to stand for 2 min. after each addition to insure sufficient time for the alkyl sulfate to react with the enzyme and allow the solution to reach a state of equilibrium before the conductivity was measured. The results of these investigations are plotted in Figs. 3 and 4.

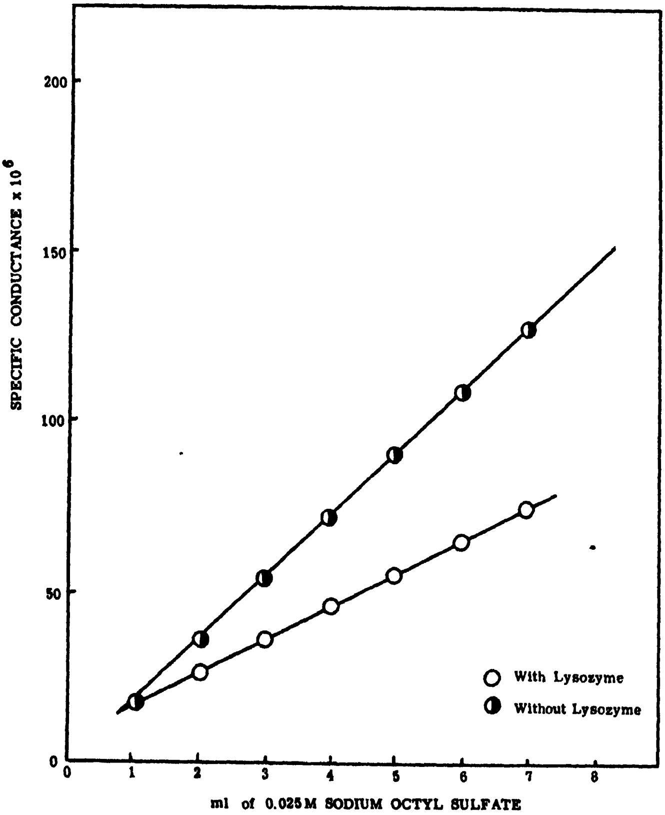

FIG. 3. A comparison in conductivity changes of lysozyme and non-lysozyme solutions when 0.025 *M* sodium octyl sulfate was added.

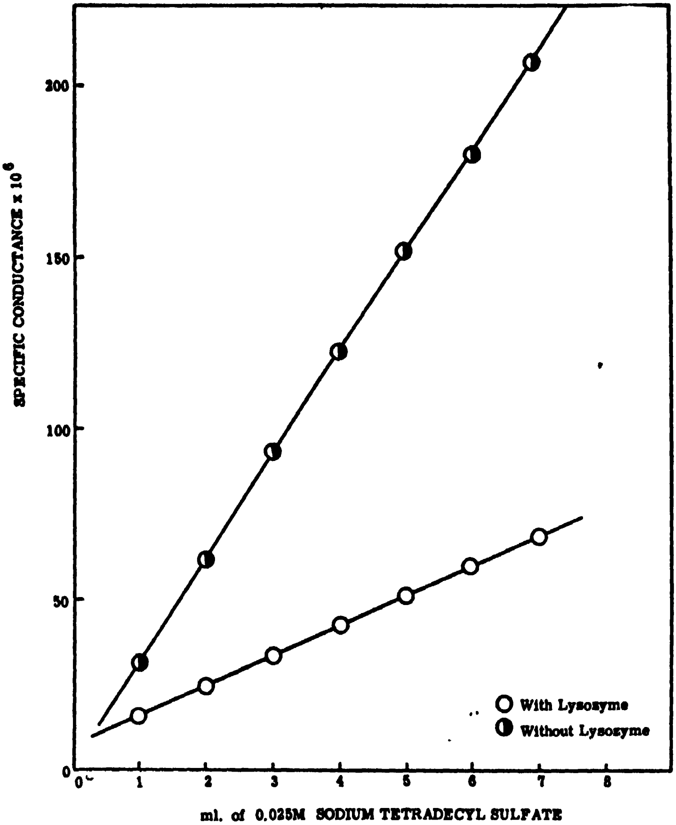

Fig. 4. A comparison in conductivity changes of lysozyme and non-lysozyme solutions when 0.025 *M* sodium tetradecyl sulfate was added.

They indicate that both octyl sulfate and tetradecyl sulfate have combined with the lysozyme in such a manner that their presence in the solution no longer contributes to the conductivity of the solution to the same extent as they did in the absence of the enzyme. This suggested that the difference between the inhibiting action of these two compounds could not be explained entirely as a difference between the two compounds on the basis of a difference in the tendency to combine with the enzyme. The length of the chain itself must be influencing the interaction of the enzyme and the alkyl sulfate which results in an inactivation of the lysozyme.

ELECTROPHORETIC MIGRATION STUDIES

The electrophoretic migration of crystalline lysozyme, lysozyme-tetradecyl sulfate complex and lysozyme-octyl sulfate complex were studied to determine whether any noticeable changes in the electrical properties of the enzyme preparation had occurred which might indicate the nature of the changes which had taken place in the protein molecule during inactivation.

The complexes were prepared by mixing 5 ml. of 0.1 *M* barbiturate buffer (pH 7.70) containing 60 mg. of crystalline lysozyme, with 10 ml. of buffer containing 150 mg. of the alkyl sulfate. The mixture was allowed to stand overnight at the temperature at which the migration was to be measured. This allowed the excess alkyl sulfate to precipitate. The precipitate was centrifuged off and discarded. Biuret tests and ultraviolet absorption studies had shown that the precipitate contained only traces of the lysozyme, and thus could be discarded. These complexes of alkyl sulfate and lysozyme, therefore, correspond to the albumin-dodecylsulfate complexes (10) in which the total acid-binding capacity of the protein has been used.

In the original experiments the rates of migration of lysozyme and the two complexes were measured at 1.5°C. At this temperature, it was possible to obtain clear cut migration patterns of the crystalline lysozyme and the lysozyme-octyl sulfate complex. With the lysozyme-tetradecyl sulfate complex, however, the migration patterns and ultraviolet absorption studies of the precipitate and supernatant indicated that most of the alkyl sulfate had precipitated at 1.5°C. and the remaining component in the supernatant was either pure lysozyme or some complex which migrates at the same rate as the crystalline lysozyme. It was found that the lysozyme-tetradecyl sulfate complex was soluble at 25°C., and electrophoretic patterns of its migration at this temperature were obtained. These patterns were compared with those obtained with crystalline lysozyme and lysozyme-octyl sulfate, also made at 25°C. At this temperature, the lysozyme-octyl sulfate tended to precipitate in the cell. This invalidates the use of these results, but the indications were that neither the material remaining in solution nor the precipitate showed any tendency to migrate. These results indicate that, if the complex had remained in solution, similar patterns as those obtained at 1.5°C. would probably have been obtained.

The crystalline lysozyme showed a mobility toward the negative pole indicating that the protein possessed a net positive charge (see Table II). The protein migrated as a uniform boundary and no evidence for inhomogeneity was found. The lysozyme-tetradecyl complex exhibited an electric mobility toward the positive pole (see Table II), and therefore the complex possessed a net negative charge. This would indicate that the tetradecyl sulfate had reacted with the lysozyme molecule in such a manner as to eliminate the positive charge possessed by the protein molecule and allow a negative charge to predominate.

TABLE II

Summary of Electrophoretic Data Obtained with Crystalline Lysozyme at Two Temperatures and Following Its Treatment with Sodium Tetradecyl Sulfate and Sodium Octyl Sulfate

All samples were run in barbiturate buffer pH 7.70

Run no.	Sample	Temp. (°C.) of run	Electric mobility ($\times 10^5$) of boundaries		
			Ascending	Descending	Mean
T-470 No. 536	Crystalline lysozyme	1.5	(+) 5.6	(+) 5.1	(+) 5.3
T-471 No. 537	Crystalline lysozyme	1.5	(+) 5.2	(+) 5.0	(+) 5.1
T-481 No. 547	Crystalline lysozyme	25.2	(+)11.8	(+)10.3	(+)11.1
T-472 No. 538	Lysozyme (60 mg.)+sodium tetradecyl sulfate (150 mg.)	1.5	(+) 5.8	(+) 4.6	(+) 5.2
			(This evidently unchanged lysozyme. Complex found to be insoluble at 1.5°C.)		
T-482 No. 548	Lysozyme (60 mg.)+sodium tetradecyl sulfate (150 mg.)	25.7	27.6	17.4	22.5
T-485 No. 551	Lysozyme (60 mg.)+sodium tetradecyl sulfate (150 mg.)	25.4	23.8	22.0	22.9
T-472 No. 539	Lysozyme (60 mg.)+sodium octylsulfate (120 mg.)	1.5	No migration.		
T-477 No. 539	Lysozyme (60 mg.)+sodium octylsulfate (120 mg.)	1.5	No significant migration. Slight splitting of boundary.		
T-483 No. 549	Lysozyme (60 mg.)+sodium octylsulfate (150 mg.)	25.3	No migration. Electrophoresis cell dark with precipitate near solution-buffer boundary.		
T-484 No. 550	Lysozyme (60 mg.)+sodium octylsulfate (150 mg.)	25.4	No migration. Dark precipitate at solution-buffer boundary.		

It should be noted, furthermore, that more than one component was present in the mixture as the original peak separated into two components, one of which has migrated while the other has remained stationary.

With the lysozyme-octyl sulfate complex no appreciable migration could be measured at 1.5°C. or detected at 25°C. This would suggest that the positive charge of the protein molecule has been neutralized by reaction with the octyl sulfate so that the complex no longer has sufficient charge to cause it to migrate in an electrical field.

The electrophoretic data from these experiments are in agreement with the data presented by Putnam *et al.* (10,11), Steinhardt (12,13,14), and others on the action of detergents on proteins. Putnam (10,11) has shown that two electrophoretic components can be obtained with mixtures of serum albumin and dodecyl sulfate. One of these components corresponds to a complex in which the total acid-binding capacity of the protein is used, while the other corresponds to a complex in which the ratio of dodecyl sulfate to protein is only half as great. This smaller component had the viscosity of serum albumin, while the fully combined component had an abnormally great viscosity.

As has been pointed out by Steinhardt (14), inactivating efficiency of the large chain compounds can probably be explained on the basis of their action in shifting the basic dissociations of the amide and peptide groups in the protein, which would allow the C-N bonds to be more easily split and which would result in a denaturation of the enzyme.

ACKNOWLEDGMENT

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SUMMARY

1. Studies have been conducted to determine the relationship between the length of the carbon chain and the functional group in various series of straight chain aliphatic compounds and the inactivation of crystalline lysozyme by these compounds.

2. Data have been presented to show that optimal inactivating efficiency occurs in a carbon chain range of C_{12} to C_{16}. In this range the inhibiting action of the compounds increases with length of the carbon chain and with concentrations.

3. Alkyl sulfates, fatty acids, sodium salts of fatty acids, and aliphatic long chain alcohols, have been found to inhibit crystalline lysozyme if they possess a carbon chain containing more than 12 carbons.

4. The solubility of the compounds tested also influences their inhibitory action. Since only the soluble component of a compound is effective in inhibiting lysozyme the more soluble the compound the more of it will be able to react with the enzyme.

5. Inactivation of lysozyme by alkyl sulfates could not be reversed by dialysis or precipitation of the inhibiting agent with barium or calcium ions.

6. Electroconductivity and electrophoretic data have been presented which indicate the inhibition is not due simply to the interaction of the inhibiting agent with a functional group of the enzyme to produce insoluble complexes but is probably caused by changes in the protein molecule itself.

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The Effect of the Soybean Trypsin Inhibitor on the Enzymatic Release of Amino Acids from Autoclaved Soybean Meal [1]

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INTRODUCTION

It has been well established that proper heat treatment or methionine supplementation will effect a substantial improvement in the nutritive value of raw soybean (1–3). Melnick *et al.* (4) have suggested that, in the case of raw soybean, methionine is liberated in the gastrointestinal tract too late to effect mutual supplementation of the other essential amino acids. Considerable attention has been directed to the presence of one or more trypsin inhibitors in raw legumes (5–10), and the deleterious effect of such inhibitor concentrates on the growth of experimental animals (11–16). The foregoing evidence would strongly suggest that the poor growth-promoting qualities of raw soybean may be attributed to an inhibition of *in vivo* enzymatic digestion by the antitryptic factor, which, according to the hypothesis of Melnick *et al.*, must specifically retard the release of methionine.

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[1] This investigation, undertaken by one of us (I. E. L.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Southern California, reports research conducted at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 224 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

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The present paper reports the results of an investigation involving a study of the effect of the soybean trypsin inhibitor on the enzymatic[4] release of amino acids from autoclaved soybean meal. Raw and autoclaved soybean meal without added inhibitor provided a suitable basis for comparison. A comparison of the effect of the trypsin inhibitor on the release of amino acids from heat-processed and from raw soybean meal is valid only if a suitable quantity of the inhibitor is added to the properly heated soybean meal to reduce its digestibility to that of the meal before heating. The initial phase of this study, therefore, deals with the determination of the level of the inhibitor necessary for its replacement in heated soybean meal to obtain a rate of digestibility substantially equivalent to that prior to heating. The second phase is concerned with the effect of this level of the inhibitor on the release of several amino acids from autoclaved soybean meal. Finally, the effect of preliminary peptic digestion on the ability of the inhibitor to retard the digestion and release of methionine from autoclaved soybean meal was determined.

EXPERIMENTAL

The soybean used was of the Bansei variety. The beans were finely comminuted and extracted with chloroform at room temperature, which reduced its fat content from 24% to 2%. The resulting meal, autoclaved at 15 lb. pressure (115°C.) for 20 min., was found to exhibit maximum digestibility and a negative test for the antitryptic factor as determined by the method of Westfall and Hauge (13). This degree of autoclaving was a satisfactory compromise among the several time-temperature relationships reported to produce maximum nutritive value (13, 17–20).

The inhibitor extract was prepared in the following manner. A 20% suspension of the unheated meal was adjusted to pH 4.2 with concentrated HCl and stirred overnight in the cold with an electric stirrer. The suspension was filtered through cheese cloth and subsequently through rapid filter paper. The filtrate was centrifuged to remove any insoluble material that precipitated upon standing in the cold. The supernatant, designated as the inhibitor extract, contained approximately 1.5 mg. N/ml. The extract was preserved under toluene in the refrigerator, and no loss in activity was observed throughout the period of study.

The Effect of the Trypsin Inhibitor on the Digestibility of Autoclaved Soybean Meal

The *in vitro* digestion technique employed was essentially that proposed by Melnick *et al.* (4, 21). The precedure, in brief, was as follows. Samples of autoclaved soybean meal weighing 13 g. (equivalent to 6 g. of protein) were accurately weighed and transferred

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[4] The term "enzymatic," as used here, refers to the proteolytic activity of pancreatin (U. S. P. purchased from Central Scientific Company, Chicago, Illinois).

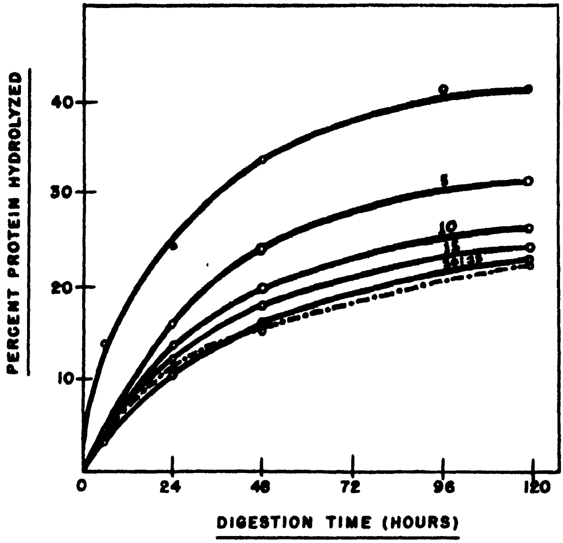

FIG. 1. The effect of increasing increments of soybean inhibitor extract on the *in vitro* digestibility of autoclaved soybean compared to raw soybean. Autoclaved soybean is represented by the smooth curve, the accompanying numerals indicating the ml. of inhibitor extract added. Raw soybean is represented by the dot-dash curve.

to 100 ml. glass-stoppered graduate cylinders. Approximately 75 ml. of borate buffer, pH 8.3, were added to each cylinder and the mixture thoroughly shaken. After being diluted to the mark with the buffer, the mixture was transferred to a 250 ml. glass-stoppered bottle. An aliquot (5–25 ml.) of the inhibitor extract diluted to 25 ml. with distilled water was added to the digestion mixture. The addition of 10 ml. of 2% pancreatin and 15 ml. of buffer brought the final volume to 150 ml. Ten ml. of toluene were added to minimize bacterial growth during subsequent incubation at 37°C. Similar mixtures of raw and autoclaved soybean meal to which inhibitor had not been added and enzyme controls containing various levels of inhibitor were run concurrently. Formol titrations were conducted on 5 ml. aliquots removed at intervals of 0, 5, 24, 48, and 120 hr. The per cent protein hydrolyzed[6] is compiled in Table I and graphically presented in Fig. 1.

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[6] Calculated by using the formula:

$$\frac{a - b}{c - b} \times 100,$$

where c = formol titration of 5 ml. of acid hydrolyzate obtained by refluxing 13 g. of soybean with 75 ml. of 8 N H_2SO_4 for 24 hr. and subsequently diluted to 150 ml.

b = formol titration at time t = 0.

c = formol titration at time t.

TABLE I

The Effect of Soybean Inhibitor Extract on the Digestibility of Autoclaved Soybean

Soybean treatment	Inhibitor extract	Digestion period	Protein hydrolysis	Inhibition	i/I[a] $\times 10^2$
	ml.	*hr.*	*per cent*	*per cent*	
None (raw)	0	5	2.1	84.0	
		24	11.6	51.7	
		48	16.1	52.1	
		120	22.7	46.0	
Autoclaved	0	5	13.2	0	
		24	24.0	0	
		48	33.7	0	
		120	42.1	0	
	5	5	4.0	69.6	7.2
		24	16.2	32.5	15.4
		48	24.1	28.3	17.6
		120	31.2	25.9	19.3
	10	5	2.6	80.4	12.4
		24	13.3	44.5	22.5
		48	20.0	40.6	24.6
		120	26.6	36.8	27.1
	15	5	2.9	78.7	19.1
		24	12.4	48.5	31.0
		48	18.3	45.5	33.0
		120	24.1	42.7	35.1
	20	5	2.4	81.8	24.5
		24	10.7	55.5	36.1
		48	16.8	50.2	39.8
		120	22.9	45.5	44.0
	25	5	1.8	86.2	29.0
		24	11.0	54.0	46.3
		48	17.1	49.3	50.7
		120	22.9	45.5	55.0

[a] Ml. inhibitor extract/% inhibition.

It will be noted from Fig. 1 that increasing increments of the inhibitor extract produced a decreasing rate of digestibility of the autoclaved soybean meal, and that 20–25 ml. of the inhibitor solution were sufficient to reduce its digestibility to approximately the same level as that of the unheated meal.

The experimental data, moreover, provided a means of verifying from a theoretical basis the level of the inhibitor extract necessary to add to the autoclaved soybean meal to obtain a digestibility equivalent to that before heating. Applying the fundamental concepts of Michaelis and Menten (22), that substrate and enzyme form an intermediate complex, and of Northrop (23), that the inhibitor competes with the substrate by combining with the enzyme in accordance with the law of mass action, an expression can be derived (24) relating the per cent inhibition of digestion (per cent decrease in the digestion of autoclaved soybean meal due to the presence of the inhibitor) as a function of the level of the inhibitor extract. This relationship can be expressed by the equation:

$$I = \frac{i}{c + k i}, \tag{1}$$

where i = ml. of inhibitor extract

I = per cent inhibition = $\frac{A - B}{A} \times 100$

A = per cent of the autoclaved soybean meal protein hydrolyzed;

B = per cent of the autoclaved soybean meal protein hydrolyzed when i ml. of inhibitor extract are added; or of raw soybean meal protein

c and k = empirical constants.

If the reciprocal of Eq. (1) is multiplied by i we obtain the expression:

$$i/I = c + ki. \tag{2}$$

Eq. (2) represents a straight line when i/I is plotted against i, the intercept on the i/I axis and the slope determining the constants c and k, respectively.

In order to evaluate the experimental data in terms of theoretical predictions, the values for i/I are included in Table I. In Fig. 2, i/I *vs.* i is plotted for the digestion periods of 24, 48, and 120 hr. Such data appeared to exhibit rather closely the linear relationship predicted by Eq. (2). The constants c and k, expressed as the intercept on the i/I axis and the slope, respectively, are recorded in Table II. Since the percent inhibition, I, exhibited by the unheated meal, is known for each digestion period (see Table I), it is now possible to calculate from Eq. (1) the corresponding concentration of the inhibitor extract, i, necessary to effect this degree of inhibition. The results of such calculations in Table II give an average value for i of 20.3, which is within the range of 20 to 25 ml. of inhibitor extract observed in Fig. 1 as being necessary to add to autoclaved soybean meal to produce the same rate of digestibility as that of the unheated protein.

The values for constants c and k were inserted into Eq. (1) in order to calculate the theoretical curves shown in Fig. 3, where percent inhibition, I, is plotted against ml. of inhibitor solution, i. Experimental data taken from Table I are likewise plotted for comparison.

The Effect of the Trypsin Inhibitor on the Release of Amino Acids from Autoclaved Soybean Meal

The rate of release of several amino acids was studied on raw and autoclaved soybean meal, and on the latter to which 25 ml. of inhibitor extract[6] had been added. The previously described digestion technique was used with the modification that, in

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[6] For the purpose of convenience it was decided to use 25 ml. of inhibitor extract, this being justified by the fact that the difference in digestibility effected by 20 or 25 ml. was negligible (see Figs. 1 and 3).

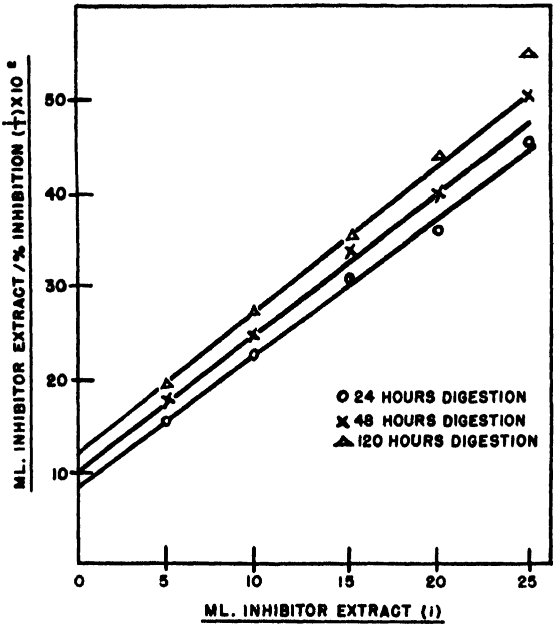

FIG. 2. Graphic determination of the constants c and k in the equation: $i/I = c + k\,i$ where c is the intercept on the i/I axis and k is the slope of the curve. (See text for details.)

TABLE II

Concentration of Inhibitor Extract Necessary to Produce a Degree of Digestibility in Autoclaved Soybean Equivalent to Raw Soybean

Digestion period	$c \times 10^2$ [a]	$k \times 10^2$ [b]	Inhibition of raw soybean[c]	i, inhibitor extract[d]
			per cent	*ml.*
24 hr.	8.5	1.47	51.7	18.0
48 hr.	10.0	1.50	52.1	23.6
120 hr.	11.5	1.57	46.0	19.2
Average	10.0	1.51	49.9	20.3

[a] Intercept on i/I axis, Fig. 2.

[b] Slope of curve in Fig. 2.

[c] Taken from Table I.

[d] Calculated from Eq. (1) in text.

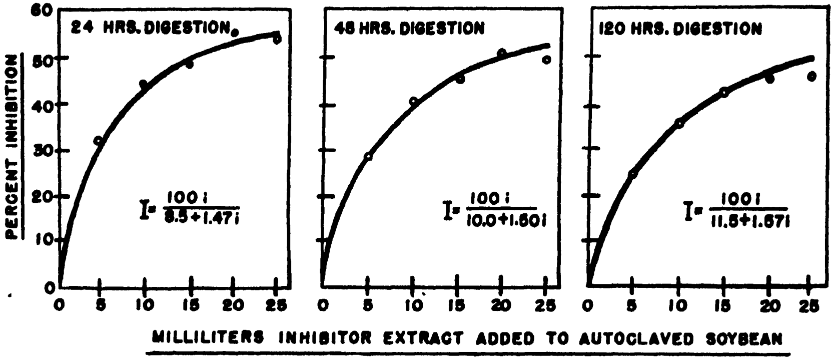

FIG. 3. A comparison of experimental data and theoretical equations based on assumption that enzyme and inhibitor form a reversible complex in accordance with the law of mass action. Curve is theoretical based on equation shown in each graph; circles represent experimental data taken from Table I.

addition to removing aliquots for formol titration at stated intervals of time, aliquots of the digestion mixture removed at the same time were placed in a steam sterilizer for 15 min. to inactivate the proteolytic enzymes. The undigested protein was precipitated by adjusting the pH to 4.2 with several drops of glacial acetic acid. The clear filtrates were preserved in the cold with toluene and subsequently used for amino acid analyses. For the latter, the microbiological techniques indicated below were so modified that the semimicro procedure of Sauberlich and Baumann (25) was applicable in all cases. *Leuconostoc mesenteroides* was used to determine aspartic acid (25), lysine (26), and methionine (27); *Lactobacillus arabinosus* was employed for the assay of isoleucine (28), leucine (29), tryptophan (30), and valine (29). The per cent amino acid released was computed by dividing the amount of the amino acid released by enzymatic hydrolysis at time *t*, by the total amino acid released by acid (in the case of tryptophan, alkaline) hydrolysis from raw or autoclaved soybean meal and multiplying by 100. The results of these assays are presented in graphs in Fig. 4.

Effect of Preliminary Peptic Digestion

The susceptibility of the crystalline soybean inhibitor to peptic inactivation has been reported by Kunitz (10). *In vitro* digestion experiments by Evans and his coworkers (31,32) showed that the proteins of raw and autoclaved soybean were digested and the methionine released to approximately the same extent when tryptic digestion was preceded by digestion with pepsin. It was deemed advisable, therefore, to determine directly the effect of preliminary peptic digestion on the enzymatic release of methionine from autoclaved soybean meal to which the inhibitor had been added, the heated and unheated meals without added inhibitor again providing a suitable basis for comparison.

The digestion procedure remained the same except that 0.1 *N* HCl and 0.1 g. of pepsin (1:10,000) were substituted for the buffer and pancreatin, respectively. After

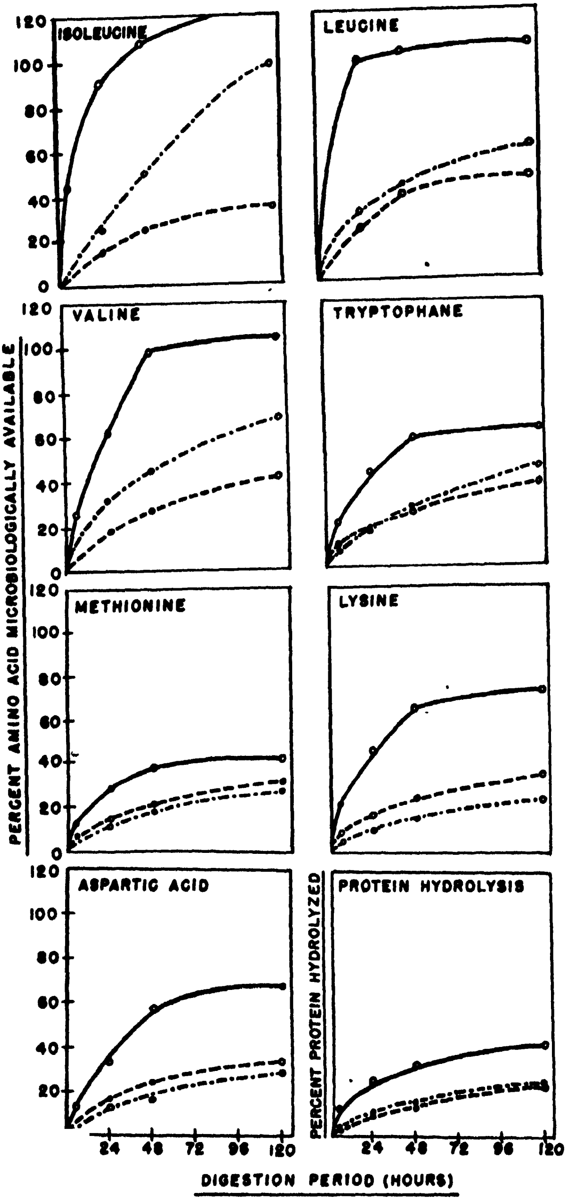

FIG. 4. Rate of release of microbiologically available amino acids from autoclaved soybean (smooth curve), raw soybean (broken curve), and autoclaved soybean plus inhibitor (dot and dash curve). Digestibility is shown for comparison.

48 hr. incubation at 37°C., the mixture was adjusted to a pH of 8–9 with a few drops of saturated NaOH. After the addition of 0.2 g. of pancreatin, followed by a further incubation period of 96 hr., formol titrations and microbiological assays for methionine were conducted. These results are tabulated in Table III.

TABLE III

The Effect of Preliminary Peptic Digestion on the Digestibility and Release of Methionine from Soybean

Method of digestion	Raw	Autoclaved	Autoclaved + inhibitor[b]
	Per cent protein digested		
Pancreatin[a]	23.6	43.0	24.2
Pepsin and pancreatin	42.8	43.0	35.7
	Per cent methionine released		
Pancreatin[a]	33.0	42.0	29.2
Pepsin and pancreatin	70.5	64.0	60.5

[a] Preliminary digestion with heat inactivated pepsin.

[b] 25 ml. of inhibitor extract.

DISCUSSION

From Fig.3 it is evident that the experimental data are in good agreement with the theoretical assumption that the mechanism of the inhibition of the digestion of soy protein by pancreatin consists in the formation of an enzyme-inhibitor complex reversibly dissociating in accordance with the law of mass action. These results, as well as those reported by Borchers *et al.* (33), are at variance with the observation by Kunitz (10), that crystalline soybean inhibitor and crystalline trypsin form an irreversible stoichiometric compound. The conditions of the experiments reported here are not quite comparable in all respects since the enzyme preparation used, pancreatin, may contain a variety of proteolytic enzymes, and the crude inhibitor may include a number of antitryptic factors (7,8) which may or may not combine with these proteolytic enzymes in the manner described by Kunitz for his crystalline materials.

In confirmation of previously reported results (4,34), Fig. 2 shows that the release of all the amino acids studied proceeded at a slower rate from the unheated meal than from the autoclaved material. There was no evidence, however, to indicate that methionine was specifically retarded in its release from the unheated soybean meal. Riesen *et al.*

(34) have also stated that . . . "heat treatment increased the rate of liberation of methionine, however, not proportionately more than it increased the rate of liberation of the other essential amino acids, excluding lysine."

Although the effect of the inhibitor extract on autoclaved soybean meal was to produce an overall rate of digestibility substantially equivalent to the raw meal (see "per cent protein hydrolyzed"—Fig. 4), the release of the individual amino acids varied above and below the level of release of the corresponding amino acids of the raw meal. If the curves of the autoclaved protein to which the inhibitor had been added are compared with the curves of the raw soybean protein in Fig. 4, it can be noted that the rates of release of lysine, in particular, and methionine and aspartic acid, to a lesser degree, are less than the corresponding rates before heating. On the other hand, the rates of release of the other amino acids, isoleucine and valine especially, are greater.

The explanation for these differences undoubtedly lies in the fact that the effect of heat on soybean is two-fold: (1) inactivation of the trypsin inhibitor, and (2) alteration of the physical properties of the soybean protein *per se* (35), which is quite independent of the inhibitor. Since, under the conditions of this experiment, the trypsin inhibitor which had been inactivated by heat is in effect replaced, any differences in the rate of release of amino acids from raw and autoclaved soybean meal to which the inhibitor had been added may be attributed to the effect of heat on the protein. In this connection, Riesen *et al.* (34) have similarly noted that soybean meal autoclaved for 4 min. released all of its essential amino acids except lysine at a faster rate than the raw meal to which sufficient pancreatin had been added to overcome the effect of the trypsin inhibitor. Under the conditions reported here, that is, autoclaving soybean meal for 20 min., methionine and aspartic acid as well as lysine become more refractory to enzymatic release; nevertheless, this same treatment still facilitated the release of leucine, isoleucine, valine, and possibly tryptophan, to a greater degree than can be explained by inactivation of the inhibitor alone. Since the release of all amino acids is adversely affected by excessive heat (34), it becomes apparent that there must exist for each amino acid a critical degree of exposure to heat, below which its release is facilitated and above which its release is retarded; whichever effect occurs, however, is usually obscured by the concomitant inactivation of the trypsin inhibitor.

It is interesting to note that the two amino acids which were observed to be most vulnerable to heat, lysine and methionine, also tend to become the first limiting amino acids causing the lowered nutritive value of overheated soybean meal (17–19).

Table III indicates that preliminary digestion with pepsin obscures the differences in digestibility and the release of methionine between raw and autoclaved soybean meal, an effect which may be ascribed primarily to the action of pepsin on the inhibitor.

Evidence has been presented in recent papers (14,15) to show that trypsin inhibitor concentrates exert their major growth-inhibiting effect through a mechanism not directly related to an inhibition of normal enzymatic hydrolysis in the intestinal tract. Such an observation might be expected if the trypsin inhibitor were destroyed or inactivated by peptic digestion in the stomach *of the rat.* It is difficult to determine to what extent our *in vitro* results can be extended to conditions *in vivo.* It may be significant to note, however, that most experiments with rats thus far reported have yielded results comparable to those obtained by serial enzymatic digestion shown in Table III, that is, the slight difference in digestibility (4,36) as well as the ultimate intestinal absorption of methionine (4,37) between raw and autoclaved soybean.[7] It is possible, therefore, that, because of peptic inactivation in the stomach, the trypsin inhibitor may be nonoperative *in vivo* and the poor growth-promoting quality of raw soybean may be attributed, at least in part, to a heat-labile substance closely associated with it. Animal experimentation is now in progress to determine the validity of this hypothesis.

SUMMARY

1. Experimental and theoretical data provide a means for determining the amount of soybean trypsin inhibitor extract to add to autoclaved soybean meal to produce a degree of digestibility equivalent to that of the unheated meal.

2. The inhibition of the digestion of soybean protein appears to involve enzyme and inhibitor in reversible equilibrium in accordance with the law of mass action.

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[7] That the trypsin inhibitor is still active in the intestinal tract of the chick was shown by Ham *et al.* (11). In contrast to rats, chicks exhibit marked differences in digestibility and absorption of methionine between raw and autoclaved soybean (32,38). It was suggested by Evans (32) that this species difference may be due to poor gastric digestion in the chick, which approximates *in vitro* digestion by trypsin and erepsin, and the higher degree of peptic digestion in the rat.

3. The release of methionine from raw soybean meal was not found to be specifically retarded by the trypsin inhibitor but followed the same general pattern of inhibition evidenced by other amino acids.

4. The antitryptic factor does not fully account for the difference in the release of amino acids from raw and autoclaved soybean meal. In addition to the inactivation of the antitryptic factor, alteration in the soybean protein as a result of heat may retard or facilitate the release of amino acids.

5. Peptic inactivation of the trypsin inhibitor masks differences in digestibility and release of methionine between raw and autoclaved soybean meal. The possible nutritional significance of these results is discussed.

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The Photochemistry of Proteins. VII. A Quantum Yield for the Inactivation of Ficin by Ultraviolet Light [1]

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INTRODUCTION

In the first papers of this series (1,2) the possibility of gaining some insight into protein structure by comparative studies of quantum yields for the inactivation-denaturation (3) of crystalline enzymes by ultraviolet light has been outlined. Early work in this field (1,3) was handicapped by the lack of well defined protein materials and means of correlating observable gross physical and chemical changes with the amount of light absorbed. Real progress in quantitative treatment has its groundwork in the papers of Uber and coworkers (4,5,6) and of Rideal and Mitchell (7,8,9) for processes occurring in solution and monolayers, respectively. Investigation of changes in enzymatic activity appeared especially promising because of a possible correlation of inactivation with chemical changes of the primary process. In view of the desirability of finding a material giving a high quantum yield for such a correlation, a systematic survey is under way in this laboratory (2,10,11,12).

No proteolytic enzyme derived from plant sources and no enzyme activated by thiol groups has been investigated heretofore.

Such a study with pure crystalline Walti-Ficin (13), practically free of ash, is the subject of this paper. Although ficin will hydrolyze gelatin and benzoylglycylamide (13) as well as benzoylargininеamide (14) without an activator, the rate of hydrolysis is slower than in the presence of a suitable activator. Furthermore, Irving, Fruton and Bergmann (14) have shown that the rate constant of hydrolysis of benzoylarginineamide (BAA) without activator, though representative of a first order rate with respect to BAA for a given enzyme concentration, is not directly proportional to enzyme concentration. For instance, it is more than doubled by doubling the enzyme concentration. This was also found true in the case of activation by HCN, but thioglycolic acid and cysteine showed first order constants proportional to enzyme concentration.

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[1] For preceding paper in this series see Finkelstein, P., and McLaren, A. D., *J. Polymer Sci.*, 1949, in press.

[2] From a thesis to be submitted to the Dept. of Chemistry, Polytechnic Institute of Brooklyn, in partial fulfillment of the requirements for the Ph.D. degree.

No quantitative study of activation of ficin by H_2S has been reported previously, but Sumner and Somers (15), as well as Balls and Lineweaver (16), and Winnick, Cone and Greenberg (17), state that such activation does take place, while Jaffé (18) includes Na_2S in his experiments on the activation of uncrystallized ficin. All the work described in this paper was done with ficin activated by H_2S, which was found to hydrolyze benzoylarginineamide by a strictly first order relationship with respect to BAA, yielding first order rate constants directly proportional to the ficin concentration (see Table I). Winnick *et al.* (17) also established the activity of ficin using casein as a substrate. Our own

TABLE I

Hydrolysis of Benzoylarginineamide by H_2S-Ficin

Enzyme conc. mg. ficin/ml.	Incubation time	Hydrolysis	K (1st order)	$K^1 = \frac{K}{E}$
	hrs.	*per cent*		
0.05	4	18	0.000824	0.0165
	8	33	0.000833	0.0167
0.0625	4	22	0.001035	0.0165
0.1	4	33	0.00167	0.0167
	2	18	0.00165	0.0165
0.125	4	38	0.00199	0.0160
0.15	4	45	0.00249	0.0166
0.1875	4	52	0.00305	0.0163
0.25	2	39	0.00411	0.0165
	1	22	0.00414	0.0166

results with a synthetic substrate confirm their findings that H_2S activates ficin, but less than does cysteine. The molecular weight of crystalline ficin, essential for the calculation of a quantum yield, has been reported by Rothen (19) as 30,000. As far as we could ascertain, no ultraviolet absorption spectrum of ficin has been published.

EXPERIMENTAL

Materials

Pure crystalline ficin was dissolved in $M/15$ KH_2PO_4 (pH 4.5). A concentration of 0.5 mg./ml., corresponding to an absorption of 68% of the total light at 2537 Å, was used for most of the work, although one series was run at a concentration of 1 mg./ml., corresponding to 91% absorption at the same wavelength. The ultraviolet absorption spectrum was taken at concentrations of 0.5 mg./ml. and 0.25 mg./ml. before and after irradiation, respectively, and the molecular extinction coefficient calculated (4,5,6) (see Table II).

The α-benzoyl-L-arginine amide hydrochloride used as substrate was prepared according to the directions of Bergmann, Fruton and Pollock (20) and dissolved in $M/15$ KH_2PO_4 to a final concentration of 0.03 mM/ml.[3] A saturated H_2S solution was used as activator. Since the solubility at room temperature is approximately 0.34 g./100 ml., this corresponds to 0.1 mM/ml.

A Hanovia Sc-2537 mercury resonance lamp with a 5000 volt-60 milliampere secondary coil transformer was used to irradiate the ficin solutions, contained in fused quartz cells 1 cm. deep and with a capacity of 4.5 ml., stirred continuously by means of a quartz stirrer operated by a small motor. Between the test solution and the arc, which was exactly 3 cm. from the face of the solution, another identical cell containing 43% acetic acid acted as a filter. (For a complete description of technique see Ref. 12.) After irradiating the solution (generally containing 0.5 mg. ficin/ml.) for definite periods, aliquots were withdrawn and the loss of ability to hydrolyze BAA determined.

Activity Determinations

A calibration curve of activity *vs.* enzyme concentration was made in the usual way (6,11). For 4 hr., varying amounts of ficin plus 0.2 ml. saturated H_2S solution plus 0.5 ml. BAA plus enough KH_2PO_4 ($M/15$) to give a total of 1 ml. were incubated at 37°C. Preliminary experiments demonstrated the optimum activator concentration to be 0.2 ml. saturated aqueous H_2S/ml. Samples of the irradiated enzyme solution, 0.3 ml., plus 0.2 ml. saturated H_2S solution plus 0.5 ml. BAA solution were also

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[3] In the icebox the benzoylarginineamide solutions will keep without decomposition for 1–2 weeks. The following simple qualitative test was, however, found advisable: On a small watch glass a drop of the solution is made into a paste with some solid magnesium oxide. A moist strip of red litmus paper is placed on the inside of an identical watchglass to be used as a lid. If the litmus paper does not turn blue (NH_3 released) on slight warming on top of a waterbath, the solution of substrate may be used.

TABLE II

Ultraviolet Absorption Spectrum of Ficin in M/15 KH_2PO_4(pH 4.5)

λ	$\epsilon = \frac{D^a}{C}$	ϵ after 10 min. irradiation
2400	60000	94800
2500	28800	56400
20	28800	55200
40	30000	55200
60	32400	56400
80	35400	58800
2600	38400	61200
40	45600	67200
80	54000	73800
2720	60000	80400
40	63000	81600
60	64200	81600
80	64500	80400
2800	63600	79400
2900	39600	52560
3050	5280	16200

[a] ϵ = molecular extinction coefficient = optical density $\log_{10}\left(\frac{I_o}{I}\right) \div$ concentration of ficin in moles/l. for a 1 cm. path length.

incubated at 37°C. for 4 hr. and the residual activity found by comparison with the calibration curve. In all cases the pH was 4.5.

The extent of hydrolysis may be determined essentially in 2 ways: (a) by titrating the released COOH groups; (b) by determining the amount of NH_3 set free. In most investigations involving benzoylargininеamide as a substrate the first named method has been applied, in particular the Grassmann and Heyde (21) microtitration technique (*e.g.*, 14,5,6). In view of the difficulty of obtaining consistent results (22) other analytical procedures were studied. Butler (23), for the same substrate, employs a modified formol titration. Formol titrations have also been used in the case of other amides (16,24) split by papaïnases. In a recent paper by Bray, James, Ryman and Thorpe (24) a comparison of this method with an ammonia aeration gave identical results. NH_3 aeration from saturated K_2CO_3 into H_2SO_4 and back titration has also been used by Geddes and Hunter (25) to estimate the hydrolysis of asparagine by asparaginase. In the hands of Hofmann and Bergmann, determination of the NH_3 liberated on enzymatic hydrolysis of benzoyllysineamide (26), α-hippuryl-L-lysineamide (26) and benzoylglycyl-L-arginineamide (27) yielded between 90 and 98% of the values obtained by titration of the COOH groups (21), while the liberation of NH_3 from benzoylarginineamide was also observed by Bergmann, Fruton and Pollock (20). In our case, the determination of the ammonia split off seemed to yield the most consistent and reproducible results. The micro diffusion technique, perfected and described in great detail by Conway (28), was found preferable to the several aeration procedures. Setting up a series of Conway dishes, both the calibration curve (Table I) and the inactivation curve (*vide infra*) (Fig. 1) were obtained by absorption of ammonia in $N/100$ HCl and nesslerization. In some cases back titrations with $Ba(OH)_2$ were used as a check, but did not give any significant improvement in accuracy. In each instance blanks were run with ficin only and with the undigested BAA solution. Potassium carbonate was used to inactivate the enzyme and to liberate NH_3. A few drops of lead acetate were introduced in the outer compartment of the Conway dish to bind the H_2S and avoid any interference in the subsequent color reaction. Since all of the incubated material was analyzed, the tubes were rinsed out with a little distilled water. The lead acetate solution present in the outer compartment of the Conway dish served as an indicator that all the H_2S, and, therefore, all the other ingredients, had been washed out. Only a small opening between the coverglass and the porcelain dish was left open to the atmosphere and, with practice, the whole procedure could be completed in a few seconds. Although the ammonia is said to diffuse in approximately 1 hr. (28), we found it more convenient to leave the dishes set up over night and remove the acid solution from the inner compartment next day for nesslerization. This assured quantitative results. To test the first order equation $K = K^1E = (1/t) \ln \left(\frac{a}{a-x}\right)$, where E is the enzyme concentration in mg. ficin/ml., a is the initial concentration of BAA and x is the amount of BAA hydrolyzed at time t, 0.3 ml. ficin solution (containing varying amounts of ficin) plus 0.2 ml. H_2S solution and 0.5 ml. BAA were also incubated. First order kinetics were observed (Table I).

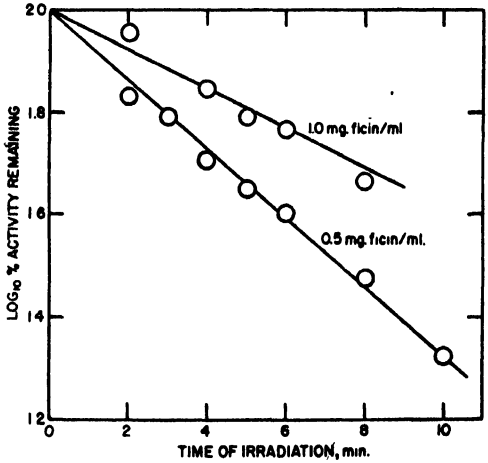

FIG. 1. Inactivation of ficin by ultraviolet light at λ2537A.

Absorption Spectra

The ultraviolet absorption spectrum of ficin in $M/15$ KH_2PO_4 (pH 4.5), before and after irradiation, were measured with a Beckmann quartz spectrophotometer equipped for the ultraviolet range with a hydrogen discharge lamp, in quartz cells 1 cm. in depth (Table II).

RESULTS AND DISCUSSION

A quantum yield, Φ, based on the loss in ability to release NH_3 from α-benzoyl-L-arginineamide can be calculated from the loss of activity *vs.* time of irradiation curve (Fig. 1) (11).

$$\Phi = \frac{\text{moles inactivated}}{\text{einsteins absorbed}} = \frac{cf}{MIF}$$

$$\text{or} \quad \Phi = 0.014 = \frac{0.50\times10^{-3}\times0.143}{30{,}000\times0.265\times10^{-6}\times0.68\times0.91}$$

where c = concentration in g. ficin/ml.,
f = fraction inactivated in 1 min. = 0.143,
M = molecular weight (19) = 30,000,
I = intensity in einsteins impinging on the contents of the cell in 1 min./ml.[4] = 0.265×10^{-6},
F = fraction of light absorbed at 2537 Å (0.68) corrected for the fraction of light absorbed on the average by active enzyme during the experiment (0.91).

The significance of this correction has been discussed elsewhere (3–6, 10–12). Briefly, at the start of the irradiation all the light absorbed is absorbed by native, active enzyme, whereas, at the end of irradiation, some of the light is absorbed by inactive enzyme. The factor 0.91 is the average fraction (evaluated by graphical integration (6)) of the absorbed light absorbed by active enzyme during the irradiation.

The interpolated value of the remaining activity after 1 min. of irradiation is used, since the light absorption changes rapidly with inactivation. This factor is apparently not of importance, since the experimental points do not drift from the curve (Fig. 1) with time. With the more concentrated solution (1 mg./ml.) the quantum yield was 0.013. This value is probably the more correct, since the fraction of light absorbed (0.91) is closer to that absorbed by the actinometer used in the intensity determination (1.0). Irradiation of ficin did not produce any odor, discoloration, or precipitate nor any other visible change, but the molecular extinction coefficient increased significantly as tabulated in Table II. The ultraviolet spectrum of ficin is analogous to that of other enzymes (5,11,29), in accord with Dhéré's (30) observation that the aromatic amino acid residues determine the selective character of protein absorption spectra. A maximum of $\epsilon = 64,500$ was observed at 2780 Å, a minimum of $\epsilon = 28,500$ at 2510 Å. Like all other enzymes investigated, the inactivation is found to follow a strictly first order law (see Fig. 1) (11).

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[4] Intensity measurement by Mr. P. Finkelstein. An uranium oxalate actinometer was employed as described in Bowen, E. J., Chemical Aspects of Light, p 283. Oxford Univ. Press, London, 1946.

TABLE III

Quantum Yield for the Inactivation of Enzymes and Related Proteins at $\lambda = 2537$ *Å*

	pH	Substrate	Φ	M. W.	Ref.
Chymotrypsin	4.65	Casein	0.0032	41,000	11
Ficin	4.5	Benzoylarginineamide	0.013	30,000	This paper
Pepsin	2.01	Hemoglobin casein	0.0025	35,500	12
Ribonuclease	—	Yeast nucleic acid	0.026	15,000	31
Soybean trypsin inhibitor	3.15	Trypsin	0.0088	24,000	10
Tobacco mosaic virus	7.00	Tobacco	0.00004	4.2×10^7	32
Trypsin	4.5	Casein Benzoylarginineamide	0.01–0.019 0.017	36,500	5,6
Urease	6.5	Urea	0.00093	483,000	4

The quantum yields for the inactivation of enzymes and related substances thus far determined, and their respective molecular weights, are summarized in Table III. It can be seen that there is a rough inverse correlation between quantum yield and molecular weight. The highest quantum yield has been obtained with ribonuclease, which has the lowest molecular weight. The molecular weight of ficin is about twice as large, and the quantum yield is about half the former. Although the proportionality is only approximate, enzymes of similar molecular weight, under comparable experimental conditions, such as pH, *etc.*, show quantum yields within an order of magnitude. This correlation seems to indicate that our search for a protein with a quantum yield of 0.1 or more must involve the investigation of proteins of much lower molecular weight as, *e.g.*, secretin (M. W. 5,000).

SUMMARY

A quantum yield for ficin of 0.013 at 2537 Å has been determined, based on loss of ability of the enzyme to hydrolyze α-benzoyl-L-arginineamide in the presence of H_2S activator. The kinetics of the action of ficin activated by H_2S on benzoylarginineamide has been investi-

gated. The ultraviolet absorption spectrum of ficin and the change in absorption after 10 min. irradiation at 2537 Å were measured. A possible relationship between the quantum yields obtained and the molecular weights of the enzymes is discussed.

ACKNOWLEDGMENT

We are greatly indebted to Dr. A. Walti of the Biochemical Division, Interchemical Corp., Union, N. J., for the crystalline ficin, prepared by him and generously placed at our disposal.

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The Properties of the Enzyme-Substrate Compounds of Peroxidase and Peroxides. I. The Spectra of the Primary and Secondary Complexes*

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INTRODUCTION

When the iron atom of horseradish peroxidase combines with hydrogen peroxide, a series of 3 distinct colored compounds is formed—green (I), pale red (II), and bright red (III). Complexes II and III were first seen, and the positions of their visible absorption bands were measured by Keilin and Mann (1). Later, Theorell (2) discovered complex I and measured the position of its visible absorption band. Theorell found that the primary complex (I) preceded the secondary (II) and tertiary (III) complexes.

In this paper, the primary and secondary complexes of horseradish peroxidase with methyl or ethyl hydrogen peroxide are reported, and the positions of their visible absorption bands are given. By means of a rapid spectrophotometric method (3), the spectra of all these unstable peroxidase-peroxide complexes have been measured in the region of the Soret band.

It is now possible to make a comparison of the properties and the spectra of 9 primary and 9 secondary hematin-peroxide complexes, for, in addition to these 6 primary and secondary peroxide complexes of horseradish peroxidase, primary and secondary compounds of catalase (4) and lactoperoxidase (5) have been studied. The occurrence and nature of the absorption spectra of these primary complexes is found to be a general property of the peroxide compounds of horseradish peroxidase, lactoperoxidase, and catalase.

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* This is No. VII of a series of papers on Catalases and Peroxidases.

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In this and the following papers, the kinetics of the enzyme-substrate compounds of horseradish peroxidase are studied in some detail in order to determine which of the 3 enzyme-substrate compounds are involved in the enzymatic activity and whether complex I or II is the rate-determining step in the oxidation of acceptors studied previously (6). Since kinetic studies are made in the region of the Soret band, a correlation between the visible and the Soret bands of these complexes is given here.

EXPERIMENTAL

Methods

A spectrophotometer (3) using an electronically stabilized light source and a sensitive and rapid amplifier and recording circuit, is employed in two ways. For slower reactions, an ordinary 1.33 cm. open cuvette is used and the peroxide is added as a drop on the end of a rod and the solution is rapidly stirred—giving mixing times of about 1 sec. For more rapid reactions, these cuvettes are replaced by a micro form of the rapid-flow method of Hartridge and Roughton and Millikan which gives mixing times of less than a millisecond (6).

Preparations

Crystallized horseradish peroxidase (7) was obtained through the generosity of Prof. H. Theorell and Dr. K. G. Paul. The alkyl hydrogen peroxide solutions were described elsewhere (8). The notatin preparation was generously supplied by Prof. D. Keilin and Dr. E. F. Hartree.

Correlation of the Visible and Soret Bands of the Primary and Secondary Complexes

On addition of an equivalent of H_2O_2 to peroxidase, the transient green color of the primary complex is seen and is soon followed by the red color of the secondary compound. The green compound has an absorption band at 655 mμ (2), and the red compound has two absorption bands at 560 and 530 mμ (1).

Using dilute peroxidase and the sensitive spectrophotometer, we have recorded the formation and disappearance of complex I in Records C and D of Fig. 1 upon adding 8 μM H_2O_2 to peroxidase in an open 1.3 cm. cuvette. Initially, the optical density of free peroxidase is recorded; then, on addition of the peroxide with stirring, a rapid density change occurs at both 411 and 635 mμ upon the formation of complex I and is complete before the stirring rod can be removed from the solution. In about a minute, complex I changes to complex II which, at these two wavelengths, has the same optical density as free peroxidase. In the records at 427 and 570 mμ, the optical density is initially that of free peroxidase. On addition of peroxide, only the slow formation of complex II is recorded. Complex II is relatively stable in distinct contrast to "cyclic" behavior of complex I. In several minutes, however, complex II spontaneously decomposes, and the optical density of free peroxidase is obtained.

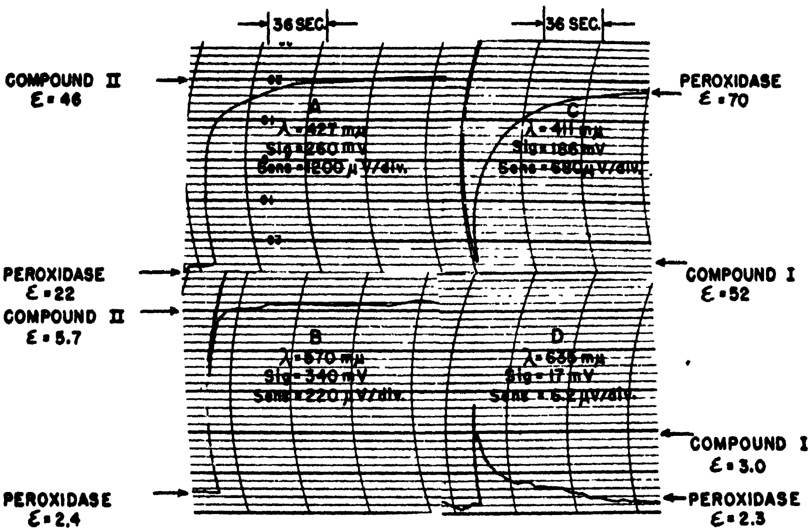

FIG. 1. A correlation between the visible and Soret bands of complexes I and II. Complex I is identified at 635 and 411 mμ (C and D) by its "cyclic" behavior, and complex II at 570 and 427 mμ (A and B) by its relatively greater stability. An upward deflection of the trace indicates an increase of optical density. These experiments were carried out in a 1.33 cm. open cuvette. The photocurrents and sensitivities at the 4 wavelengths are indicated. 8 μ*M* H_2O_2 was added to the same 2.9 μ*M* horseradish peroxidase solution after the liberation of the enzyme from the previous test. The units of ε are $cm.^{-1} \times mM^{-1}$. pH = 4.5, 0.01 *M* acetate buffer (Expt. 168).

These records identify complex I and complex II as having isosbestic points with peroxidase at 427 and 411 mμ, respectively. These records do not, however, show that the kinetics of disappearance of complex I are identical to the kinetics of appearance of complex II, since several additions of peroxide intervened between Records A and B and C and D. These kinetics are studied quantitatively later (10).

With about 100-fold stronger peroxidase and visual spectroscopy, the band of complex I at 660 mμ[2] formed on addition of H_2O_2 is still seen, although the color of the solution has changed from greenish to pale red (see Table 1). Simultaneous spectrophotometric measurements show that there is only a small change in the extinction coefficients at 560 and 655 mμ corresponding to this color change. Theorell (11) also has observed this phenomenon—"only a blend of the green type (I) and the first red type (II) could be produced for magnetic measurements." Apparently, it is difficult to obtain pure complex II in strong peroxidase solutions. On the other hand, the formation of complex III causes the disappearance of both complex I and II as shown in Table 1.

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[2] The band of Complex I appears to lie at 655–660 mμ.

TABLE I

Visual and Spectrophotometric Data on the Compounds of Peroxidase and Hydrogen Peroxide

240 μM horseradish peroxidase pretreated with H_2O_2, pH = 7.0, 0.005 M phosphate buffer (Expt. 308)

Compounds		I+(II)	(I)+II	III
Added peroxide—mM		0.20	0.20	100
Time after addition of peroxide—min.		1	5	—
Visible spectrum	Color	Greenish	Pale red	Red
	bands—mμ	530-560- 660(strong)	530-560- 660(faint)	545 582
Spectrophotometric data	Wavelength—mμ	560 655 680	560 655 680	560 655 680
	Extinction coefficient—$cm.^{-1} \times mM^{-1}$	5.7 4.1 2.3	5.7 3.8 2.1	6.3 2.3 2.3

Visual Spectroscopy and Spectrophotometry of the Compounds of Peroxidase and Alkyl Hydrogen Peroxides

Since it has been shown previously that catalase forms green and red complexes with the alkyl hydrogen peroxides (4,8) and that peroxidase has appreciable activity with ethyl hydrogen peroxide (12), the existence of peroxidase-alkyl hydrogen peroxide complexes was readily verified. The addition of a few equivalents of methyl hydrogen peroxide to strong peroxidase (230 μM) gives the transient green color of complex I which rapidly changes to a nearly black mixture of the green (I) and red (II) complexes with three bands at 530, 560, and 660 mμ. Further addition of methyl hydrogen peroxide gives a pale red color due to a preponderance of complex II. The absorption band of compound I is, however, still visible at 660 mμ. In these two complexes, the use of alkyl-hydrogen peroxide gives the same effects as H_2O_2. But a large excess of methyl hydrogen peroxide does not give a red compound (III) with bands at 545 and 580 mμ as in the case of H_2O_2 (1); with methyl hydrogen peroxide in the course of about 5 min., the band at 660 mμ strengthens, shifts to 675 mμ, and becomes very intense giving the solution a beautiful green color. The bands of complex II at 560 and 530 mμ are replaced by a single band at 557 mμ. This complex is stable for at least 1 hr. On addition of hydroquinone, the green complex slowly decomposes—much more slowly than if the same amount of H_2O_2 instead of methyl hydrogen peroxide had been added. Only a fraction of the original peroxidase is recovered, since much of the peroxidase is destroyed by the excess peroxide. Thus, this is a new peroxidase-peroxide complex which has no known analogue in the peroxidase-hydrogen peroxide complexes nor in the catalase-peroxide complexes. Therefore, this compound will be designated complex IV to avoid confusion.

These spectroscopic data are summarized in Table II. Also Fig. 2 shows in curve B the spectrum of a mixture containing a preponderance of compound I and in curve C the spectrum of the new compound IV.

TABLE II

Visual and Spectroscopic Data on the Compounds of Peroxidase and Methyl Hydrogen Peroxide

230 μM horseradish peroxidase, pH = 7.0, 0.005 M phosphate buffer (Expts. 304, 306, 309)

Compounds		I+(II)	(I)+II	IV	IV plus a few crystals of hydroquinone
Added methyl hydrogen peroxide-mM		0.25	3.0	180	
Time after addition of peroxide-min.		1.0	23	5	20
Visible spectrum	Color	Dark green	Pale red	Bright green	Brownish
	bands—mμ	530 560 660 (strong)	530 560 660 (faint)	557 675 (weak) (very strong)	640(faint)
Spectrophotometric data	Wavelength-mμ	560 655 680	560 655 680	560 655 680	560 655 680
	Extinction coefficient cm.$^{-1}$×mM^{-1}	5.3 3.4 1.7	6.2 3.0 1.5	4.0 7.1 6.9	3.5 2.2 1.7

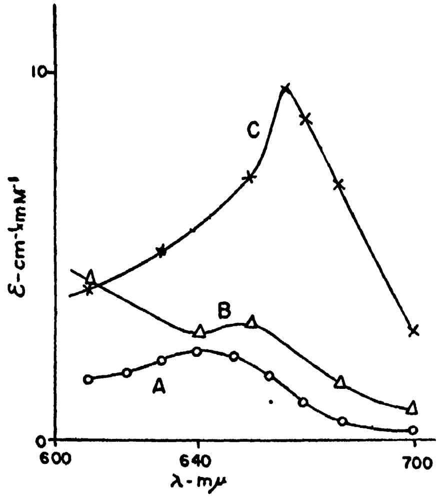

FIG. 2. Curve A is free peroxidase; B, a mixture of complexes I and II (430 μM peroxidase, 1 mM CH_3OOH); C, complex IV (230 μM peroxidase, 120 mM CH_3OOH) 0.01 M phsophate, pH = 7 (Expt. 304).

The Spectra of the Secondary and Tertiary Peroxidase-Peroxide Complexes in the Soret Region—the Hydrogen Peroxide Complexes

With dilute peroxidase (3 μM), the spontaneous decomposition of the secondary complex is slow enough at pH = 4.7 to permit single readings in the Beckman spectrophotometer immediately after adding several equivalents of H_2O_2, but not sufficiently slow to permit readings over the whole spectrum. The extinction coefficient of the secondary complex was found to be $\epsilon_{417} = 77$ cm.$^{-1}$ × mM^{-1} at pH = 4.7 on the basis of a number of determinations. The complete spectrum was determined by repeated additions of peroxide to the peroxidase solution. This method was not entirely satisfactory because of the danger of accumulating an excess of peroxide and forming compound III. Satisfactory results were obtained using H_2O_2 continuously generated by notatin, glucose, and oxygen (9), and the Soret spectrum of Complex II is plotted in Fig. 3. It is seen to agree at 417 mμ with the value obtained using "bottle" H_2O_2. As the H_2O_2 accumulates, the spectrum of compound III is obtained.

The extent to which the Soret band of complex III falls below that of complex II depends upon the peroxide concentration; with a large excess of peroxide (~30 mM), the band may fall to one-third of its initial height in 15 min. owing to the destruction of peroxidase.

As later tests show, complex II is more stable in dilute alkaline phosphate buffer, and its spectrum has been measured with the ordinary spectrophotometer in the region 370–450 mμ and substantial agreement with the curve of Fig. 3 is obtained. The height of the Soret band at pH = 7.9 appears to be about 5% greater than at pH = 4.6.

The very large peroxidase concentrations required for visual spectroscopic tests gave in several cases mixtures of the primary and the secondary complexes, and it is desirable to ensure that these spectra obtained with dilute peroxidase represent the pure secondary compound. With a large excess of acceptor (ascorbic acid), the primary complex is quantitatively converted into the secondary complex in a fraction of a second, and the secondary complex is stable for several seconds (see (10), Fig. 6). The spectrum of the secondary complex under these conditions can be obtained in the rapid-flow apparatus by converting the recorder deflections into changes of extinction coefficient from free peroxidase to peroxidase-H_2O_2-II according to the following equation. This equation is valid for the flow apparatus where the optical densities involved are much less than 0.01.[3]

$$\Delta\epsilon_{\lambda_1} = \frac{0.434}{C_k} \times \frac{S_k}{E_{\lambda_1}} \times \frac{D_k}{d_o} \text{ cm.}^{-1} \times \text{m}M^{-1},$$

---

[3] This equation is derived from the usual equation

$$\epsilon = \frac{\log \frac{I_o}{I}}{C_k d_o},$$

by substituting $\log \frac{I_o}{I} = 0.434 \frac{I_o - I}{I} = 0.434 \frac{\Delta I}{I}$ for small densities and $\Delta I \alpha S_k D_k$, $I \alpha E_{\lambda_1}$. Also since ϵ is the increment from peroxidase to complex II, it is termed $\Delta\epsilon$.

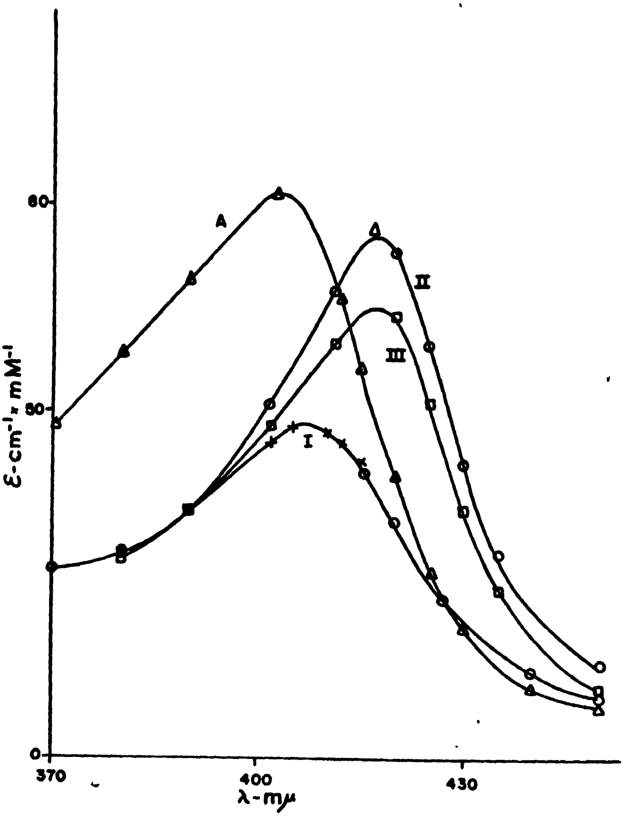

FIG. 3. The Soret bands of peroxidase (A) and complexes I, II, and III (so labeled). Point Δ of curve II was obtained soon after the addition of H_2O_2 (10 μ*M*) to peroxidase (4 μ*M*) in an ordinary spectrophotometer (Expt. 268). Curves II and III were obtained in an ordinary spectrophotometer in the presence of 6.7 m*M* glucose and about 0.03 μ*M* notatin (Expt. 269 a and b). Curve III was obtained under the same conditions as Curve II but after more H_2O_2 had accumulated. pH = 4.7, 0.1 *M* acetate (Expt. 269).

Curve I was obtained by means of the rapid flow method. Points x, 0.98 μ*M* peroxidase, 1.7 μ*M* H_2O_2 (Expt. 301). Points o, 2.6 μ*M* peroxidase, 20 μ*M* H_2O_2 (Expt. 163 d). pH = 4.7, 0.01 *M* acetate.

where C_k = Concentration of peroxidase used in the kinetic experiment (m*M*).
S_k = The sensitivity of the oscillograph in the kinetic experiment (μV/mm.).
E_{λ_1} = The voltage drop across the photocell load resistor (proportional to photocurrent) (μV) at λ_1.
D_k = The maximum oscillograph deflection in the kinetic experiment (mm.) at λ_1.
d_o = The effective diameter of the observation tube (cm.).

The value of d_o has been determined by calibrations with solutions of known optical density and is found to be fairly constant over the region 370–450 mμ at about 0.070 mm.[4]

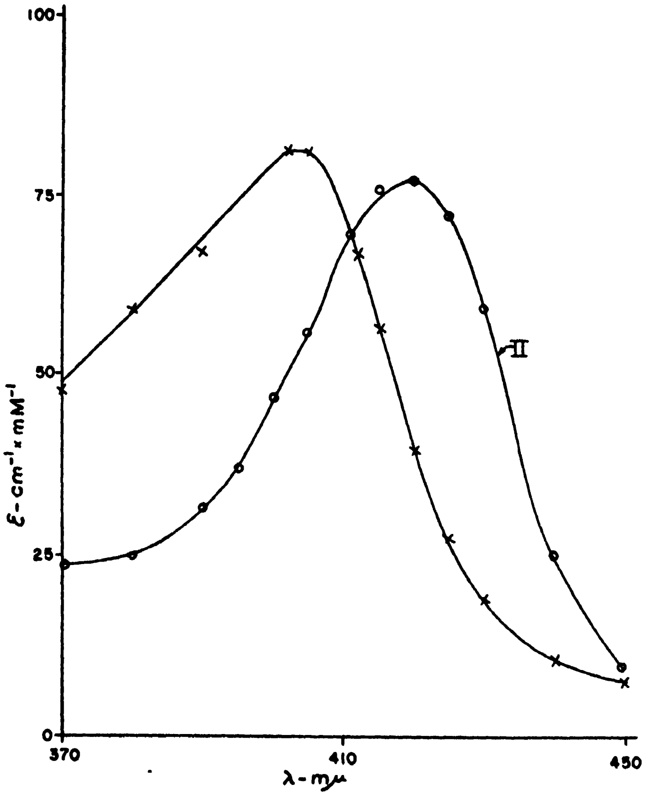

FIG. 4. The Soret band of complex II during the oxidation of ascorbic acid using the rapid-flow apparatus. Half-time for decomposition of complex II = 4 sec. 2.6 μ*M* peroxidase, 8 μ*M* H_2O_2, 100 μ*M* ascorbic acid. pH = 4.7, 0.01 *M* acetate (Expt. 166).

The changes of extinction coefficient obtained by Eq. 1 are applied as corrections to the peroxidase spectrum, and the spectrum of the secondary peroxidase-peroxide complex is obtained in Fig. 4. There is substantial agreement between this spectrum and that of Fig. 3 since the somewhat broader spectral interval (about 7.5 mμ) of the monochromator explains the broader spectrum of Fig. 4. Thus the spectrum shown in Fig. 3 represents that of the pure secondary compound.

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[4] In an earlier formula (13), d_o was not expressed explicitly because it was considered necessary to calibrate its optical depth at each wavelength. In the first formula (p. 241), the term $\frac{D_s S_s}{\epsilon_c}$ is equal to $\frac{C_k d_o E_{\lambda_1}}{0.434}$ and in the second formula (p. 242), the term $\frac{(D_k)_{\lambda_1}}{E_{\lambda_1}} \frac{(S_k)_{\lambda_1}}{(\Delta\epsilon)_{\lambda_1}}$ is equal to $\frac{C_k d_o}{0.434}$.

The Secondary Peroxidase-Alkyl Hydrogen Peroxide Complexes

The spectra of the secondary compounds of peroxidase and methyl or ethyl hydrogen peroxide are readily obtained in the Beckman spectrophotometer upon the addition of an excess of the alkyl hydrogen peroxide, since there is no possibility of forming compound IV under these conditions, and complex III does not exist. The Soret band of the secondary methyl hydrogen peroxide complex is shown in Fig. 5 and is clearly almost identical to the Soret band of the secondary hydrogen peroxide complex. Table III shows that the spectrum obtained with ethyl hydrogen peroxide is almost

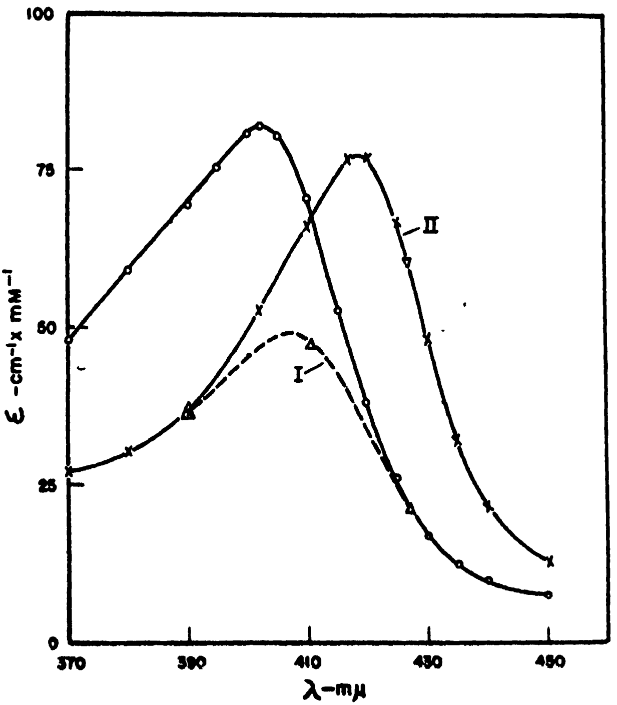

FIG. 5. The Soret band of complexes I and II of peroxidase and methyl hydrogen peroxide. Points x obtained with ordinary spectrophotometer using 2 μM peroxidase and 7 μM CH_3OOH. pH = 4.7, 0.01 M acetate (Expt. 174). Point ∇ obtained using the rapid-flow apparatus, 2.6 μM peroxidase, 20 μM CH_3OOH. pH = 4.7, 0.01 M acetate (Expt. 173a). Points Δ obtained with rapid-flow apparatus using 2.6 μM peroxidase, 4 μM CH_3OOH, pH = 6.5, 0.01 M phosphate (Expt. 177 b).

TABLE III

A Comparison of the Soret Bands of Secondary Peroxidase-Alkyl Hydrogen Peroxide Complexes

5 μM peroxidase; pH = 4.7, 0.01 M acetate (Expt. 174)

Peroxide	Conc. (μM)	λ-mμ	370	380	390	395	400	405	410	415	420	425	430	440	450
Methyl	36	ϵ-cm.$^{-1}\times$mM^{-1}	31	33	39	44	51	58	66	75	75	68	49	22	11
Ethyl	26	ϵ-cm.$^{-1}\times$mM^{-1}	28	29	38	42	50	57	66	75	77	69	49	22	11

identical with that obtained with methyl hydrogen peroxide.

In the presence of a large excess of alkyl hydrogen peroxide ($\sim$5 mM), complex II is converted into complex IV. The general characteristics of the Soret band of complex IV are very similar to those of Complex I, the peak of the band is at 410 mμ, and the shape is like that of free peroxidase. There is a continuous decrease in the intensity of the Soret band owing to destruction of the peroxidase by the excess peroxide. Thus, the extinction coefficient at 410 mμ may be much less than that of complex I. A similar effect is obtained with ethyl hydrogen peroxide.

The Primary Peroxidase-Hydrogen Peroxide Complex

The transition from the primary to the secondary complex is much too rapid to allow the use of an ordinary spectrophotometer, and the rapid-flow method has been used to obtain the values of $\Delta\epsilon$ according to Eq. 1. Since complex I is momentarily stable before disappearing into complex II, values of $\Delta\epsilon$ are obtained corresponding not only to the change from peroxidase to complex I, but also from complex I to complex II. The spectra of both peroxidase and complex II have already been obtained and the values of $\Delta\epsilon$ are applied to the appropriate curves, those from complex I to complex II (x) being more accurate from 380 to 412 mμ and those from peroxidase to complex I (o) being more accurate from 412 to 450 mμ as shown in Fig. 3.

The Primary Peroxidase-Methyl Hydrogen Peroxide Complex

This spectrum is identical with that obtained with H_2O_2 to within the accuracy of the method. Therefore, only the 3 most accurately determined values (at the three isosbestic points) are shown in Fig. 5. The dashed curve is from Fig. 3. The spectrum of the primary peroxidase-ethyl hydrogen peroxide complex is identical with that shown in Fig. 5 to within the experimental error.

DISCUSSION

The Soret spectra of the primary peroxidase-peroxide complexes are isosbestic with the spectra of the secondary complexes from 370 to 395 mμ. At this wavelength, the extinction of the primary complex rises slightly, and then falls to give an isosbestic point with peroxidase at 427 mμ. On the other hand, the extinction coefficient of the secondary complexes rises to form a Soret band of about the same height as that of the free enzyme. There is an isosbestic point between peroxidase and the secondary complexes at 411 mμ.

The spectra of the primary complexes are strikingly similar to those of the primary catalase peroxide complexes. In the visible region, both are green and have an absorption band in the red. In the Soret region, both spectra are similar in shape to the spectrum of the free enzyme and are shifted only slightly toward the visible region with respect to the free enzyme spectrum. In both catalase and peroxidase, there is a large decrease in the intensity of the Soret band on formation of the primary complexes; quantitatively, the extinction coefficient of the Soret band of catalase diminishes about 45 $\text{cm.}^{-1} \times \text{m}M^{-1}$ per hematin iron group bound to peroxide (8), and about 35 and 47 (5) $\text{cm.}^{-1} \times \text{m}M^{-1}$ for horseradish and lactoperoxidase, respectively, which have only one hematin-iron group per molecule.

Thus, there is a remarkable resemblance between the spectra of the primary compounds of catalase and peroxidase with peroxides, despite the differences in their protein components.

The analogy between the spectra of the secondary compounds of catalase and peroxidase with peroxides is more remote. Although the peaks of the Soret bands of these secondary complexes are shifted about the same amount with respect to the free enzyme spectra, the extinction coefficient of the Soret band of the secondary peroxidase-peroxide complexes is about equal to that of the free enzyme, while that of the secondary catalase-peroxide complexes is only about half that of the free enzyme. In fact, the depression of the Soret band on formation of peroxidase peroxide-III suggests that this complex is analogous to catalase peroxide-II. This view is strongly supported by kinetic data, which show that peroxidase peroxide-II is directly involved in the enzymatic activity, whereas neither peroxidase peroxide-III nor catalase peroxide-II is involved in the normal activity; the latter has been definitely proved to be an inhibitor of catalase activity.

The spectrum of neither complexes I nor II is altered by the substitution of an alkyl group on the H_2O_2 molecule and it may be concluded that this spectral similarity is a general rule in hematin-peroxide compounds. In a particular case, advantage has been taken of this rule to compute the number of H_2O_2 molecules bound to the catalase in the primary catalase-H_2O_2 complex, and the results of this computation are in accord with the results obtained by independent methods (14).

The valence of iron in all the peroxidase-peroxide complexes has been established as trivalent by the consistently negative results in attempting to inhibit the activity of peroxidase by CO (15) or in attempting to observe the ferroperoxidase-CO compound upon the addition of peroxide in an atmosphere of CO (1).

Abrams, Altschul and Hogness (16) have suggested that the spectral analogy between the visible bands of the red cytochrome c peroxidase-H_2O_2 complex and oxymyoglobin and oxyhemoglobin indicates that these red peroxidase complexes may be ferrous-oxygen compounds. However, this spectroscopic analogy fails in the region 370–450 mμ where the Soret bands of oxymyoglobin and oxyhemoglobin (17) are distinctly different from that of the cytochrome c peroxidase complex—and from that of the secondary horseradish peroxidase-peroxide complex. In this region, there is some resemblance between the relation of the Soret bands of ferrimyoglobin to oxymyoglobin and peroxidase to complex I. But these spectral analogies do not give results which are consistent in the visible and Soret regions.

The relationships between absorption spectra and variation in iron bonding which were established by Theorell (10) and were recently summarized by Hartree (18) indicate that the secondary peroxidase-peroxide complexes should have covalent bonds, by spectral analogy with peroxidase cyanide. The latter has been found to be a ferric compound with essentially covalent bonds by direct measurements of magnetic susceptibility (11). Actually, the visible and Soret bands of the secondary peroxidase-peroxide complexes and the peroxidase cyanide compound are very similar as shown by Keilin and Mann (1) and by a comparison of curves II of Figs. 3 and 5 with curve C of Fig. 6. Measurements of the magnetic susceptibility (χ_m) of a mixture of the primary and secondary peroxidase-H_2O_2 complexes (11) have been found to give $\chi_m \geq 4800$ as would be expected from a mixture of a covalent secondary complex ($\chi_m \approx 3000$) and either free peroxidase or an ionic primary complex ($\chi_m \approx 15{,}000$).

Theorell has assumed that the primary peroxidase-H_2O_2 complex has ionic bonds because both it and peroxidase fluoride, which has been found to have ionic bonds, are green. But this color analogy does not hold for the Soret bands. A comparison of curve B of Fig. 6 with curves I of Figs. 3 and 5 shows the great dissimilarity of the spectra of peroxidase fluoride and the primary peroxidase peroxides.

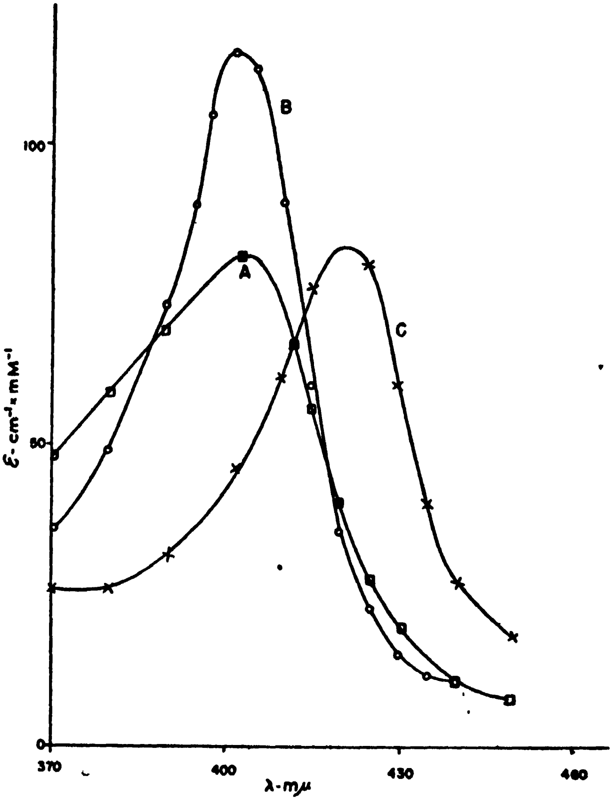

FIG. 6. The Soret bands of peroxidase-fluoride and peroxidase cyanide. Curve B, 1.6 m*M* fluoride, curve C, 33 μ*M* cyanide. 1.8 μ*M* peroxidase, pH = 4.7, 0.01 *M* acetate (Expts. 284 and 285).

The general effect upon the Soret bands of the hematins upon the formation of the primary peroxide compounds is a large diminution of intensity accompanied by a rather small spectral shift. It has been suggested that there may be interaction between the iron-peroxide complex and the methene bridges of the porphyrin ring (8). In the primary complexes such changes are apparently reversible. Complex IV has a spectrum similar to that of the primary complexes but some peroxidase is destroyed during the existence of complex IV. Complex IV is therefore similar to the "verdo" compounds studied by Lemberg in the oxidative destruction of the porphyrin groups of hemoglobin or catalase (19). However, complexes I and IV are not identical; their visible spectra differ significantly and their reaction kinetics are totally different.

It is strange that complex IV has not yet been positively identified in the reaction of peroxidase with H_2O_2. This may be due to the fact that a large excess of H_2O_2 causes the formation of the bright red complex III. However, Theorell (personal communication) has observed the slow appearance of a sharp band at 675 mμ in mixtures of the primary and secondary peroxidase-H_2O_2 complexes and has noted the destruction of peroxidase under these conditions. The absorption band may indeed be caused by a peroxidase-H_2O_2-IV which, as stated above, may be a "verdo" compound.

Summary and Conclusions

1. The spectrum of the labile green primary compound of peroxidase and H_2O_2 first seen by Theorell (2) in the hand spectroscope has been obtained in the region of the Soret band by means of the rapid-flow apparatus.

2. The spectrum of the more stable red secondary peroxidase-H_2O_2 complex first seen by Keilin and Mann (1) has been obtained by several methods, by means of the rapid-flow apparatus, and by the ordinary spectrophotometer using "bottle" H_2O_2 or H_2O_2 produced by notatin, glucose, and oxygen.

3. The primary and secondary compounds of peroxidase and methyl or ethyl hydrogen peroxide have been found, and their spectra have been obtained in the region of the Soret band.

4. These three primary peroxidase-peroxide complexes are green and have nearly identical spectra in the Soret region. Their maxima are at 407 mμ, and their spectra are isosbestic with peroxidase at 427 mμ. Their spectra are strikingly similar to those of the primary green catalase-peroxide complexes; in these primary complexes, the Soret band of the free enzyme is considerably diminished in intensity and slightly shifted towards the visible region. According to Theorell, these are ferric compounds with ionic bonds.

5. The spectra of the secondary peroxidase peroxides in the Soret region have about the same intensity as the spectrum of the free enzyme but are shifted 15 mμ to the visible. The spectra are isosbestic with peroxidase at 411 mμ at pH 4.6 to 7.0. The intensity of the absorption band of the secondary peroxidase-peroxide complex is some-

what greater at alkaline pH. According to Theorell, these are ferric compounds with covalent bonds.

6. The identity of the Soret bands of the peroxidase complexes with H_2O_2 and methyl or ethyl hydrogen peroxide, indicates that the nature of the iron-peroxide bond is unaffected by the substitution in the H_2O_2 molecule.

7. There is only gross similarity between the Soret spectra of the secondary peroxidase and catalase peroxide complexes; peroxidase-peroxide-III bears a closer resemblance to the secondary catalase peroxides.

8. In the presence of a large excess of methyl hydrogen peroxide, peroxidase forms no compound analogous to peroxidase-hydrogen peroxide-III; a bright green complex with a very strong band at 675 mμ and a faint band at 557 mμ is formed. This complex has a Soret band resembling that of the primary complexes. There is considerable destruction of peroxidase at the large peroxide concentrations required to form this complex, and it is suggested that this complex is related to the green oxidation products of hemoglobin and catalase studied by Lemberg and his associates.

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The Digestion of Acetyl Proteins by Pancreatin

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INTRODUCTION

Acetyl groups attached to the protein molecule may be divided into two classes; those hydrolyzed by treatment with dilute alkali at room temperature, and those which are resistant to such treatment. It is believed that the alkali-labile groups are attached by ester linkage involving the hydroxyl groups of the protein molecule, and that the alkali-resistant acetyl is joined by means of an amide linkage. Evidence for these linkages was obtained by Hendrix and Paquin (1), who showed that the acid-binding capacity of the protein, which was markedly diminished on acetylation with acetic anhydride, was not restored by treatment with dilute alkali. It was considered as proven that the alkali-resistant acetyl was combined with the basic groups of the protein.

The purpose of this study was the investigation of the behavior of acetyl proteins and their alkali-treated derivations toward the digestive action of pancreatic extract, and to study further the binding of the acetyl group to the protein molecule.

EXPERIMENTAL

Casein, egg albumin, and edestan were used. The casein was prepared by the method of Van Slyke and Baker (2); the egg albumin was crystallized by the method of Kekwick and Cannan (3); and the edestan was a commercial preparation (Pfanstiel "edestin"). The acetyl proteins were prepared by the method of Hendrix and Paquin. A portion of each preparation was used to prepare an alkali-treated actyl protein according to their method. The acetyl determinations were made by the method of Hendrix and Paquin and the acetyl content calculated in terms of ml. of 0.1 *N* acetic acid/g. of protein nitrogen; unacetylated proteins were used in blank determinations. It may be mentioned at this point that the ratios of nitrogen to acetyl groups in these acetyl protein preparations are essentially the same as in those preparations made by Dr. Paquin in this laboratory some years ago.

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[2] A 4% suspension of Merck's pancreatin in 50% glycerol was allowed to stand for 4 hr. after preparation, and the undissolved material was centrifuged off. The supernatant liquid was employed as pancreatin solution in the digestions.

The digestions were carried out as follows: four 1 g. samples of each of the untreated acetylated, and alkali-treated acetylated proteins were placed in 150 ml. Erlenmeyer flasks. To each sample were added approximately 1 g. of precipitated $CaCO_3$, 50 ml. of water, and about 10 drops of toluene. The flasks were then allowed to stand for 24 hr. to wet the protein thoroughly. Five ml. of a "pancreatic extract"[2] were added to each sample and all the samples were incubated at 37°C. for 48 hr. At this time an additional 5 ml. dose of the pancreatic extract was added to each of the samples and the digestion continued for an additional 48 hr. period. The digests were than transferred to volumetric flasks and diluted to exactly 100 ml. A 50 ml. portion was removed from each flask and used for the determination of the amount of acetate liberated during the digestion. The flasks containing the remaining portions of the digests were removed to the cold room to suspend enzyme action, and the contents filtered. The amino acid content of an aliquot of each filtrate was determined by a formol titration similar to that of Northrop (4). The extent of digestion of each preparation was calculated in terms of per cent of total nitrogen set free as primary amino nitrogen during the digestion. The relative digestion of the acetyl and alkali-treated acetyl proteins was calculated also by taking the extent of digestion of the unacetylated protein as 100%.

DISCUSSION

The data from these determinations are found in Table I. The acetyl casein and acetyl edestan were digested to a lesser extent than the unacetylated proteins; in terms of the amino nitrogen set free the relative digestion was slightly less than two-thirds as much as in the unsubstituted proteins. The digestion of the alkali-treated acetyl proteins was only slightly more extensive than that of acetyl casein and acetyl edestan. These results are in accord with those of Kawai (5), Yoshimatsu (6), Kisel and Roganooa (7), Dunn and Lewis (8), and Imai (9), who found that acylated, methylated, or deaminized proteins were digested by pancreatin to a lesser extent than the native proteins. It appears that the alteration of proteins in any of these ways hinders the action of some important enzyme of the pancreatic extract. Since the digestibility of acetyl casein and acetyl edestan differs only slightly from that of their alkali-treated derivatives, it would appear that any inhibition of digestion by the acetyl groups is due to those which are firmly bound, presumably those bound to the amino or other basic groups of the protein molecule. It is of interest to note that a most im-

TABLE I

Digestion of Acetyl Protein

Protein	Nitrogen	Acetyl 0.1 *N*/g. N	Acetyl lost by action of dilute alkali	Amino N freed by tryptic digestion		Acetyl freed by tryptic digestion	
				Compared to total N of protein	Compared to digestion of native protein	Compared to total acetyl	Compared to acetyl before alkali treatment
	per cent	*ml.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Native casein	13.43	—	—	51.3	(100)	—	—
Acetyl I Casein	13.33	92.0	—	35.5	69.2	29.9	29.9
Acetyl II Casein	13.11	91.6	—	35.5	69.2	34.6	34.6
Acetyl III Casein	13.96	93.1	—	34.1	66.3	30.8	30.8
Alkali-treated Acetyl Casein I	13.61	49.2	46.6	38.8	75.5	7.5	4.0
Alkali-treated Acetyl Casein II	13.78	44.7	51.5	40.2	78.4	10.9	5.3
Alkali-treated Acetyl Casein III	13.35	48.4	47.5	36.6	71.1	6.8	3.4
Native Egg Albumin	13.58	—	—	55.8	(100)	—	—
Acetyl I Albumin	13.41	141.0	—	1.75	3.13	2.9	2.9
Acetyl II Albumin	13.33	141.4	—	1.09	1.95	3.1	3.1
Alkali-treated Acetyl Albumin I	13.76	38.1	73.0	39.0	69.6	0.8	0.2
Alkali-treated Acetyl Albumin II	13.81	39.0	72.6	40.3	70.04	0.0	0.0
Edestan	15.96	—	—	54.3	(100)	—	—
Acetyl Edestan I	15.72	76.5	—	32.2	59.1	38.5	38.5
Acetyl Edestan II	15.51	75.8	—	32.9	60.6	31.3	31.3
Alkali-treated Acetyl Edestan I	15.77	19.4	74.4	34.5	63.5	2.7	0.7
Alkali-treated Acetyl Edestan II	16.13	25.8	65.9	35.4	65.2	5.21	1.78

portant fact common to the various methods of transforming the proteins used in the studies of the digestibility mentioned above, is that the ϵ amino group of lysine is masked or destroyed. Table I shows that less of the acetyl is liberated from acetyl casein and acetyl edestan by pancreatic digestion than is removed by the solution of these proteins in dilute NaOH. The acetyl removed from alkali-treated acetyl proteins by pancreatin is slight, in no case more than about 5% of the acetyl bound to the protein before treatment with enzyme solution or with alkali. The results reported in Table I give neither direct nor indirect evidence of the manner in which this small amount of acetyl was bound to the protein molecule.

The action of pancreatic extract upon acetyl egg albumin was very slight, both with respect to the amount of acetyl hydrolyzed and to the amount of digestion as shown by formol titration. As shown in Table I, less than 10% as much digestion of acetyl egg albumin occured as in case of the other proteins. Alkali-treated acetyl egg albumin was digested to about the same extent as the other alkali-treated acetyl proteins.

It is known that N-acyl derivatives are not hydrolyzed by any pancreatic enzyme except carboxypeptidase; and the acetyl derivatives of lysine, arginine, and histidine which may be presumed to account for most of the nitrogen-bound acetyl in acetyl proteins, do not fulfil the specificity requirements for this enzyme, which requires that the amino group of such a peptide linkage be an α amino group. The peptide linkage is not acetylated, even under drastic conditions of treatment, so that such a binding need not be considered as being responsible for the partial inhibition of action of pancreatic extract upon these acetyl proteins. However, some group or groups are apparently still bound, even in alkali-treated acetyl proteins, for these proteins are not as completely digested as might have been expected. In fact, the digestion of alkali-treated acetyl proteins, as shown by the formol titration, was not much greater than the digestion of acetyl casein and acetyl edestan. When the above observations are considered in connection with the fact that only very small amounts of acetyl were split from the alkali-treated acetyl proteins during digestion and that the amounts split off from the acetyl proteins during digestion were in every case less that the amount removed by treatment with dilute alkali, it appears that the enzymes responsible for the liberation of acetic acid are esterases of the pancreas which are known to hydrolyze esters of quite diverse constitutions, and that the acetyl split from the acetyl protein is, in fact, that which had been bound by oxygen linkage to such groups in the protein molecule as the phenolic hydroxyl of tyrosine, to certain other hydroxyamino acids and to the carbohydrate groups. The slight hydrolysis of acetyl actually encountered in the digestion of the alkali-treated acetyl proteins may be due to the failure of alkali treatment to remove the last traces of the oxygen-linked acetyl from the acetyl protein, but our data give no information on this question.

Qualitative work was done to show the extent of liberation of the tyrosine hydroxyl during the digestion of the acetylated proteins with pancreatic extract. Pancreatic digestion of diacetyltyrosine and dibenzoyltyrosine shows the liberation of the phenolic group in the tyrosine by the action of the enzymes. The diacetyltyrosine was prepared by the method of Bergman and Stern (10) and the dibenzoyltyrosine by the method of Schultz (11). L-Tyrosine was used in preparation of these compounds. It seems probable that no great amount of racemization occurred during the acetylation or benzoylation. Since these compounds were used for qualitative experiments only, no special attempt was made to purify them. The nitrogen contents of both these compounds were only slightly lower than the theoretical amount.

TABLE II

Millon's Reaction of Digests

Substrate	Amount	Enzyme	Incubation	Test	Remarks
O-N-diacetyl-tyrosine	200 mg.	Pancreatin 10 ml. active solution	48 hr.	++++	
O-N-diacetyl-tyrosine	200 mg.	Pancreatin 10 ml. solution heated	48 hr.	T[a]	Enzyme heated on boiling water bath 90 min.
O-N-dibenzoyl-tyrosine	500	Pancreatin 10 ml. active solution	48 hr.	++++	
O-N-dibenzoyl-tyrosine	500	Pancreatin 10 ml. heated	48 hr.	T[a]	Enzyme heated on boiling water bath 90 min.
O O	0	Pancreatin	90 hr.	T[a]	
Acetyl casein	1 g.	Pancreatin 10 ml. active solution	90 hr.	+++	
Acetyl egg albumin	1 g.	Pancreatin 10 ml. active solution	90 hr.	+?	
Acetyl edestan	1 g.	Pancreatin 10 ml. active solution		+++	

Trace.

Table II gives these results in detail. These results show the digestion and liberation of the phenolic group in all substrates, although the results obtained on acetyl egg albumin are not as conclusive as in case of other substrates. This finding is in agreement with results reported above. Table II gives the results of these qualitative experiments.

SUMMARY

Acetyl casein and acetyl edestan are digested by pancreatic extract about two-thirds as extensively as the unsubstituted proteins, under similar conditions of digestion; alkali-treated acetyl casein and edestan are digested to a comparable, but slightly greater extent, under the same conditions.

Considerable amounts of acetyl are split from acetyl casein and acetyl edestan; only small amounts were split from the alkali-treated acetyl proteins.

The hydrolysis of the acetyl group from acetyl proteins appears to be due to the action of pancreatic esterases on the oxygen-linked acetyl in the acetyl proteins. The results of qualitative experiments which are described, support this conclusion.

Acetyl egg albumin is only slightly hydrolyzed by pancreatic extract; alkali-treated acetyl egg albumin is digested by the enzyme mixture to an extent comparable to the digestion of acetyl casein and acetyl edestan. None of the results presented here explain the resistance of acetyl egg albumin to digestion.

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Structural Requirements of Specific Substrates for Chymotrypsin. II. An Analysis of the Contribution of the Structural Components to Enzymatic Hydrolysis [1]

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INTRODUCTION

Studies on the specificity of chymotrypsin have demonstrated that this enzyme hydrolyzes external and internal peptide linkages of tyrosyl and phenylalanyl peptides at the grouping which involves the carbonyl residue of the aromatic amino acids (1–3). Subsequent studies have shown that chymotrypsin, like trypsin and carboxypeptidase (4,5), hydrolyzes structural analogs of specific peptides which contain an ester group in place of the terminal amide linkage[3] (6,7), and that a positive charge on the substrate in the immediate vicinity of the susceptible peptide or ester bond decreases the affinity of the compound for the enzyme.

In the preceding paper of this series it was found that a secondary peptide group in substrates for chymotrypsin serves as a point of interaction between enzyme and substrate (9). In the present paper, a more detailed analysis of the contribution of each structural component of a specific substrate to combination with, and hydrolysis by, chymotrypsin is attempted. The major part of this investigation was carried out with specific ester substrates (4,6,7). The results have been interpreted in terms of kinetic concepts advanced on several previous occasions (7,9,10). An analogous study on the influence of structure on inhibitors for chymotrypsin is in progress (11) and will be described in full elsewhere.

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[1] Part of a thesis submitted by Mr. Seymour Kaufman to the Graduate School of Duke University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Postdoctoral Fellow of the United States Public Health Service, National Institutes of Health.

[3] The interchangeability of amino and hydroxyl groups in enzymatic reactions has been demonstrated by Blanchard *et al.* (8), who have shown that L-amino acid oxidase can also oxidize *l*-hydroxy acids.

EXPERIMENTAL

Enzyme

The preparation (No. IV) of chymotrypsin was the same as that previously described (4).

Substrates

Benzoyl-L-Tyrosineamide (BTA), Glycyl-L-Tyrosineamide (GTA), Benzoyl-L-Tyrosine Ethyl Ester (BTEE), Acetyl-L-Tyrosine Ethyl Ester (ATEE), and Carbobenzoxyglycyl-L-Tyrosineamide (CGTA) were prepared as previously described (7).

Benzoyl-DL-Methionine Ethyl Ester (BMEE). Benzoyl-DL-methionine was prepared according to the method of Carter and Stevens (12). Unless otherise stated, all benzoylations were carried out according to this method. M. p. found, 151–152°C., reported (13), 143–145°C. Esterification was carried out in the presence of absolute ethanol saturated with HCl gas. Unless otherwise stated, all esterifications were carried out in this manner. After two recrystallizations from butanol, M. p., 73–74°C.

Calculated: N, 4.98; found: N, 4.89.

Benzoyl-DL-Threonine Ethyl Ester (BThEE). Benzoyl-DL-threonine was recrystallized from water. M.p. found, 144–145°C., reported (14), 147–148°C. The ethyl ester was recrystallized from toluene. M.p., 165–166°C.

Calculated: N, 5.58; found: N, 5.26.

Acetyl-DL-Threonine Methyl Ester (AThME). Acetyl-DL-threonine was prepared according to the method of du Vigneaud and Meyer (15). The acetyl derivative was obtained as an oil. Crystallization was achieved by concentration of the alcoholic solution of the oil *in vacuo*, repeating this process several times, and allowing the syrup to stand for one week at − 10°C. The crystalline compound was esterified in the usual manner. The resulting oil crystallized after standing at − 10°C. M. p., 110–113°C.

Calculated: N, 7.86; found: N, 7.76.

Benzoyl-DL-Serine Ethyl Ester (BSEE). Benzoyl-DL-serine was recrystallized from water. M. p. found, 167–169°C., reported (16), 171°C. The ethyl ester melted at 85–86°C.

Calculated: N, 5.89; found: N, 5.60.

Phthalyl-DL-Phenylalanine Methyl Ester (PhPME). Phthalyl-DL-phenylalanine was prepared according to the method of Billman and Harting (17). M. p. found, 177–178°C., reported (12), 174–175°C. The methyl ester was recrystallized from methanol-water. M. p. 96–97°C.

309.3 $C_{18}H_{15}O_4N$ Calculated: C, 69.60; H, 4.85; N, 4.53. Found: C, 69.60; H, 4.47; N, 4.63.

Ethyl-α-Acetaminocinnamate (EAAC). This compound was prepared from the corresponding azlactone (18). The azlactone was suspended in benzene and treated with a solution of sodium ethylate according to the method of Carter and Risser (19). The ester was obtained as an oil which crystallized readily in the cold. It was recrystallized from toulene-ethyl ether. M. p., 95–96°C.

233.3 $C_{13}H_{15}O_3N$ Calculated: C, 66.90; H, 6.43; N, 6.01. Found: C, 67.09; H, 6.30; N, 6.00.

Benzoyl-DL-Phenylalanine Ethyl Ester (*BPEE*). Tthe benzoyl derivative was recrystallized from ethanol-water. M. p. found, 185–186°C., reported (20), 187–188°C. The ester was recrystallized from ethanol-water. M. p. found, 94–95°C., reported (20), 95–95.5°C.

Calculated: N, 4.71; found: N, 4.56.

Benzoyl-L-Phenylalanine Ethyl Ester (*BPEE*). This compound was prepared in the same way as the racemate. M. p., 101.5–102°C.

Calculated: N, 4.71; found: N, 4.63.

Ethyl-DL-α-Benzylacetoacetate (*EBAA*) was prepared according to the general method of acetoacetate condensation (21). The reaction mixture was fractionated at reduced pressure through an 8 inch Vigreaux column. The fraction which boiled at 175–185°C. at 30–31 mm. pressure was collected. B. p. (atmospheric pressure) found, 276–277°C., reported (22) 276°C. The dinitrophenylhydrazone was prepared. M. p. found, 69–71°C., reported (23), 71.5°C.

N-Benzoyl-o-Nitro-L-Tyrosine Ethyl Ester (*BNTEE*). *o*-Nitro-L-tyrosine nitrate was prepared according to the method of Johnson and Kohmann (24). The nitrate was benzoylated and the benzoyl derivative was recrystallized from about 10% ethanol. M. p., 179–182°C. The ethyl ester was obtained in the form of long, canary yellow needles. M. p., 158.5–159°C.

358.3 $C_{18}H_{18}O_6N_2$ Calculated: C, 60.5; H, 5.02; N, 7.81. Found: C, 60.6; H, 5.14; N, 7.86.

N-Benzoyl-DL-Phenylalanyl-O-(N-Benzoyl-DL-Serine Ethyl Ester) (*BPBSEE*). Four g. of recrystallized benzoyl-DL-phenylalanine were finely ground and dried at 75°C. for 6 hr., and subsequently in a vacuum desiccator over P_2O_5 for 4 hr. The dried material was suspended in ether and 25 cc. of acetylchloride added while the mixture was cooled and protected from moisture. Three and one-half g. of PCl_5 was added and the mixture shaken for 15 min. in the cold, and then for 0.5 hr. at room temperature. Petroleum ether was added, the mixture shaken and the ether siphoned off. The white residue of benzoyl-DL-phenylalanyl chloride was dissolved in 100 cc. of chloroform. To this was added 2.7 g. of benzoyl-DL-serine ethyl ester in 100 cc. of chloroform, and the homogeneous solution was refluxed for 1.5 hr. Considerable quantities of HCl gas evolved. The solution was concentrated *in vacuo*, yielding a yellow oil. After shaking with petroleum ether, removal of the ether *in vacuo*, and storage at − 10°C., crystallization occurred. M. p. (with softening), 152°C.

488.5 $C_{28}H_{28}O_6N_2$ Calculated: C, 69.0; H, 5.72; N, 5.72. Found: C, 69.8; H, 5.60; N, 5.57.

Benzyl Diethylmalonate (*BDEM*). This compound was prepared according to the general method of malonic acid condensation (25). The organic fraction was distilled through a Vigreaux column at 183–188°C. at 23–24 mm. pressure. The half-ester (EBM) was obtained according to the method of Marguery (26). The potassium salt was obtained as a very hygroscopic white solid. Electrometric titrations: 0.310 g. of the salt, dissolved in 10 cc. of 50% methanol, and titrated with 0.199 *N* NaOH.

Equivalent weight: Calculated: 260.0; found: 263.0.

Ethyl-DL-α-Benzaminophenylacetate (EBAPA). Eastman DL-α-amino-phenylacetic acid was benzoylated. M. p. found, 170–171°C., reported (27), 175.5°C. The benzoyl derivative was esterified by refluxing with absolute ethanol in the presence of a small amount of concentrated HCl. M. p., found, 81–83°C., reported (27), 84°C.

Calculated: N, 4.95; found: N, 4.89.

Acetyl-DL-Tryptophan Ethyl Ester (ATrEE). N-acetyl-DL-tryptophan (Winthrop) was esterified by refluxing with absolute ethanol in the presence of concentrated HCl. M. p., 137–138°C.

274.3 $C_{15}H_{18}N_2O_3$ Calculated: C, 65.5; H, 6.55; N, 10.2. Found: C, 65.7; H, 6.47; N, 10.1.

Enzymatic Measurements. Unless otherwise stated, measurements were carried out out at 25°C. in the presence of 0.045–0.050 *M* phosphate buffer, pH 7.8, containing 30% methanol. Amidase and esterase activities were determined with the methods previously described (4). Enzyme and substrate solutions were freshly prepared for each experiment.

RESULTS

Measurements of the rate of hydrolysis were interpreted according to the theoretical considerations previously described (7,9,10). This included determination of the order of reaction at constant initial substrate concentration, and evaluation of the enzyme-substrate dissociation constant, K_m, and of the specific rate constant k'. The latter is a measure of the rate of activation of the enzyme-substrate complex/mg. of enzyme N/cc./min. (9). Hydrolysis of all substrates described herein followed first order reaction kinetics to at least 50–60% of initial substrate concentration. Initial substrate concentration of specific ester substrates was determined by interpolation according to a method previoulsy described (4,7). K_m and k' were determined from a plot of one of the equations of Lineweaver and Burk (28), *i.e.*,

$$\frac{a}{v} = \frac{K_m}{V_{\max}} + \frac{1}{V_{\max}} a. \tag{1}$$

where a is the substrate concentration in moles/l., v the initial reaction velocity, expressed as the moles/l. of substrate hydrolyzed during the first min. of hydrolysis under the influence of a constant enzyme concentration of e mg. of enzyme N/cc., and $V_{\max}$ the maximum velocity prevailing when the substrate concentration is so high that all of the enzyme is tied up in the enzyme-substrate complex. $V_{\max}$ is equal to $k'e$, where k' is the specific rate constant of the activation of the stable enzyme-substrate, as already defined. K_m is the enzyme-substrate dissociation constant and is numerically equal to the substrate concentration (moles/l.) at which the initial velocity, v, is one-half of the maximum velocity, V_{max}. These kinetic relations have been considered in detail on several previous occasions (7,9,10). Presentation of experimental data from which the kinetic constants were obtained, will be limited to two novel substrates, *i.e.*, those containing tryptophyl and methionyl residues (ATrEE and BMEE). The corresponding constants for the other substrates which were tested in this work were evaluated in exactly the same manner.

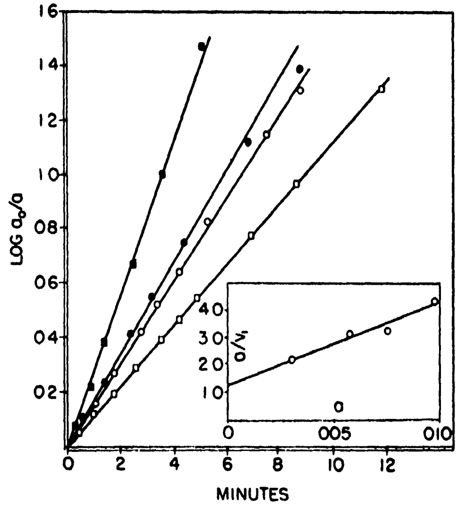

Fig. 1. Hydrolysis of acetyl-DL-tryptophan ethyl ester by chymotrypsin (9.92×10^{-3} mg. of enzyme N/cc.) plotted according to first order reaction kinetics. Initial substrate concentration was as follows: □ = 0.0099 M, ○ = 0.0076 M, ● = 0.0058 M, ■ = 0.0030 M. In the inset graph, text Eq. (1) is plotted for the determination of K_m and V_{max}, where v is the moles/l. of substrate hydrolyzed during the first min. of hydrolysis.

Acetyl-DL-Tryptophan Ethyl Ester (*ATrEE*). Hydrolysis of this substrate proceeded to 50% of completion with respect to the initial concentration of the racemate, indicating that only one of the two isomers was hydrolyzed by chymotrypsin. In accordance with the established antipodal specificity of chymotrypsin toward other subtrates which contain a secondary peptide bond (1–3), it may be assumed that only L-ATrEE was hydrolyzed. Quantitative rate measurements are shown in Fig. 1, in which, according to the first order reaction equation, log a_0/a is plotted against time. Although linear relations were obtained for each initial substrate concentration, the slope of these lines decreased with increasing initial substrate concentration, as previously shown for other enzyme-substrate systems (7,10). Evaluation of K_m and k' from the plot of Eq. (1), shown in the inset graph of Fig. 1, yielded values of K_m = 0.0048, and k' = 0.363. From these constants, the maximum proteolytic coefficient, $C_{max} = \frac{k'}{2.3\ K_m}$ was calculated[4] as 33.

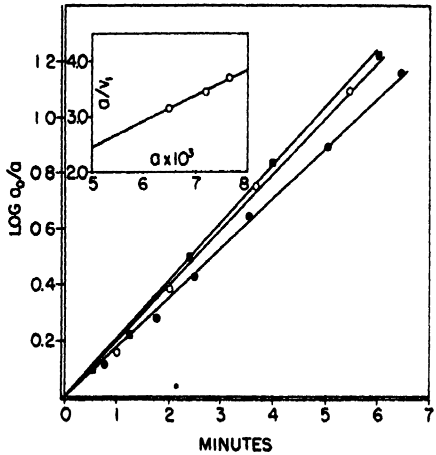

FIG. 2. Hydrolysis of benzoyl-DL-methionine ethyl ester by chymotrypsin (2.86×10^{-1} mg. of enzyme N/cc.), plotted according to first order reaction kinetics. Initial substrate concentration was as follows: ● = 0.00765 M, ○ = 0.00715 M, ■ = 0.00649 M. In the inset graph, text Eq. (1) is plotted for the determination of K_m and V_{max}, where v is the moles/l. of substrate hydrolyzed during the first min. of hydrolysis.

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[4] For the derivation of this equation, see (10). The factor 2.3 in the denominator arises from the fact that the proteolytic coefficient C (first order reaction constant/mg. of enzyme N/cc.) as defined by Bergmann and coworkers was based on the use of $\log_{10}$ instead of natural logarithms in the calculation of first order reaction constants. It has been retained herein to facilitate comparison of the present data with those of Bergmann and coworkers (1–3). The dimensions of C_{max} are min.$^{-1}$ × mg.$^{-1}$ × cc.

TABLE I

Susceptibility of Structural Analogs of Specific Substrates to Chymotryptic Hydrolysis[a]

Compound		Abbrev.	Split[b]	K_m	k'	C_{max}
No.	Name					
1	Glycyl-L-tyrosineamide[g]	GTA	[c]	0.122	0.0041	0.015
2	Benzoyl-L-tyrosineamide	BTA	[c]	0.0424	0.0065	0.066
3	Benzoyl-L-tyrosine ethyl ester	BTEE	[c]	0.0039	0.82	91.
4	Acetyl-L-tyrosine ethyl ester	ATEE	[c]	0.074	4.5	26.
5	Carbobenzoxyglycyl-L-tyrosineamide	CGTA				0.006[h]
6	N-Benzoyl-o-nitro-L-tyrosine ethyl ester	BNTEE	[c]	0.0114	3.4	128.
7	Benzoyl-L-phenylalanine ethyl ester	BPEE	[c]	0.0057	0.39	30.
8	Benzoyl-DL-methionine ethyl ester	BMEE	[c]	0.00083	0.008	4.2
9	Acetyl-DL-tryptophan ethyl ester	ATrEE	[c]	0.0048	0.363	33.
10	Benzoyl-DL-serine ethyl ester	BSEE	[f]			
11	Benzoyl-DL-threonine ethyl ester	BThEE	[f]			
12	Acetyl-DL-threonine ethyl ester	AThEE	[f]			
13	Benzoyl-DL-phenylalanyl-O-(benzoyl-DL-serine ethyl ester)	BPBSEE	[e]			
14	Ethyl-DL-α-benzaminophenylacetate	EBAPA	[e]			
15	Ethyl-α-acetaminocinnamate	EAAC	[d]			
16	Ethyl-α-benzylacetoacetate	EBAA	[f]			
17	Tyrosine ethyl ester	TEE	[e]			
18	Benzyl diethylmalonate	BDEM	[f]			
19	Ethyl-α-benzylmalonate	EBM	[f]			
20	Phthalyl-DL-phenylalanine methyl ester	PhPME	[f]			

[a] Unless otherwise stated, measurements were carried out at 25°C., in 0.045 *M* phosphate buffer, pH 7.8, containing 30% methanol. For definition and discussions of the kinetic constants, see the text.

[b] The symbols in this column denote the relative magnitudes of susceptibility of the substrates to enzymatic hydrolysis, as follows:

[c] For esters: Complete hydrolysis in not more than 30 min. by about 0.05–0.001 mg. of enzyme N/cc.

[c] For amides: Complete hydrolysis in not more than 2 hr. by about 0.1–0.2 mg. of enzyme N/cc.

[d] For esters: Not more than about 15% hydrolysis in 30 min. by about 0.2–0.3 mg. enzyme N/cc.

[e] For esters: 2–3% hydrolysis in 30 min. by 0.2–0.3 mg. of enzyme N/cc.

[f] For esters: No measurable hydrolysis in 30 min. by 0.2–0.3 mg. of enzyme N/cc.

[g] Measurements in aqueous solutions in the absence of methanol, 0.1 *M* phosphate buffer, pH 7.8.

[h] Proteolytic coefficient at 0.025 *M* initial substrate concentration.

Benzoyl-DL-Methionine Ethyl Ester (BMEE). Data analogous to those presented for ATrEE, are shown in Fig. 2. With this substrate, too, hydrolysis occurred to 50% of completion of the initial concentration of the racemate, indicating exclusive activity of chymotrypsin toward the L-isomer. From the data given in Fig. 2, the following kinetic constants were evaluated: $K_m = 0.00083$, $k' = 0.0080$, and $C_{\max} = 4.2$.

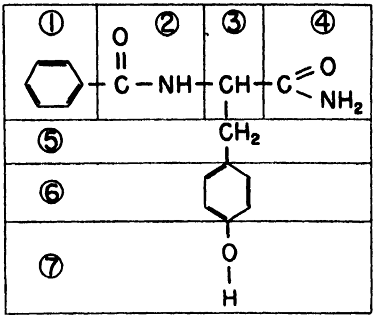
Benzoyl-L-Phenylalanine Ethyl Ester (BPEE). The hydrolysis of this ester in 30% methanol likewise followed first order reaction kinetics. The calculated reaction constants were: $K_m = 0.0057$, $k' = 0.39$, and $C_{\max} = 30.0$. Subsequent studies on the chymotryptic hydrolysis of the methyl ester of benzoyl-L-phenylalanine (9) in 20% methanol yielded comparable values for these constants.

N-Benzoyl-o-Nitro-L-Tyrosine Ethyl Ester (BNTEE). The following constants characterize the hydrolysis of this chromogenic ester substrate by chymotrypsin: $K_m = 0.0144$, $k' = 3.4$, and $C_{\max} = 128$.

Other Compounds. Qualitative measurments of the hydrolytic effect of chymotrypsin on the remaining compounds listed in the experimental section of this paper, indicated the rates of hydrolysis to be too low to be measured in quantitative terms. Whereas, in the quantitative measurements on the preceding ester substrates, and those previously described (6,7), hydrolysis by approximately 0.05-0.001 mg. of enzyme N/cc. went to completion within not more than 30 min., with the present compounds, 0.2–0.3 mg. of enzyme N/cc. produced, at the most, 15% of hydrolysis in 30 min. Because of this extremely low order of magnitude of hydrolysis, the results obtained with these compounds are merely expressed in qualitative terms by the symbols defined at the bottom of Table I. These results are given in Table I in which, for purposes of comparison, the quantitative data for the hydrolysis of the considerably more reactive substrates of the present and preceding studies (7) are also given.

DISCUSSION

A discussion of the significance of the results of the present investigation is facilitated by a diagrammatic resolution of a typical substrate for chymotrypsin into its structural components. This is shown below for the typical substrate benzoyltyrosineamide.



The principal structural constituents are: the benzamino group (comprising components 1 and 2 of the above diagram), the tyrosyl residue (components 5–7), and the amide group (component 4). In the following discussion, the contribution of each structural component (components 1–7 of the above diagram) will be considered separately.

Component 1 (Secondary Phenyl Group)

Replacement of the phenyl ring by a methyl group causes a 5.5-fold increase in the specific activation rate of the enzyme-substrate complex (compare k' for BTEE and ATEE, respectively). In accordance with the considerations set forth in the preceding paper on this subject (9), it may be suggested that the increased activation rate is caused by a better orientation of the substrate which contains the methyl group (ATEE) in place of the phenyl ring (BTEE). This would imply a higher affinity and lower enzyme-substrate dissociation constant, K_m, for ATEE as compared to BTEE. However, it has already been pointed out (9) that this constant can only serve as an index for the relative affinities of two substrates for the same enzyme if the change in K_m is greatly disproportionate to that in k', whereas, in the present case, the enzymatic hydrolysis of the compound with higher K_m is also characterized by a higher k'. The relative affinities of these two compounds for chymotrypsin are thus indeterminate.[5]

Component 2 (Secondary Peptide Group)

It has already been demonstrated that replacement of this group by a positively charged amino group greatly decreases the susceptibility of the resulting compound to chymotryptic hydrolysis (7). This is further corroborated by the present, more detailed data, which show that K_m of GTA in aqueous solutions is about 3 times higher than that of BTA in 30% methanol. Since the addition of methanol has no effect on the reaction constants, k', of certain substrates for chymotrypsin,[6] whereas it greatly increases K_m (about 5- to 7-fold in the presence of 30% methanol), it is evident that, under identical external conditions, K_m of GTA is some 15–20 times higher than K_m of BTA.[7]

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[5] Determinations of the thermodynamic constants for the chymotryptic hydrolysis of BTEE and ATEE, now under way, should yield qualitative comparison of the relative affinities of these two substrates for the enzyme. Similar comparison of the corresponding amides, *i.e.*, BTA and ATA is to be included in this analysis.

In a preceding discussion of the influence of the secondary peptide bond on the susceptibility of substrates to chymotryptic hydrolysis (9) it was suggested that this bond serves as a point of attachment by hydrogen bond formation. The results obtained in the present work provide additional evidence for this hypothesis, and indicate that a hydrogen donor at the site of the secondary peptide group is required:

1. If the *nitrogen* of the secondary peptide group is eliminated, as in ethyl-*dl*-α-benzylacetoacetate (EBAA, Compound 16 of Table I), which is the analog of the specific substrate benzoyl-L-phenylalanine ethyl ester, no enzymatic hydrolysis occurs, presumably because the keto form of this ester is incapable of forming hydrogen bonds by donation. While the enol form of this compound (29) possesses a hydrogen donating center, the α hydrogen atom and free rotation between the α and β carbon atoms have been sacrificed. Either or both of these structural changes may be unfavorable for enzyme-substrate combination.

2. Elimination of the *hydrogen* atom from the secondary peptide bond of acetylphenylalanine ethyl ester yields ethyl-α-acetaminocinnamate (EAAC, Compound 15 of Table I), the dehydro analog of the parent ester. The very slow enzymatic hydrolysis of this compound may be related to the equilibrium which exists among various keto-enol and *cis-trans* isomers (30), only one of these having the requisite structure for combination with chymotrypsin. Since only the enol form has a hydrogen-donating grouping, it may be suggested that the equilibrium concentration of the *cis* or *trans* isomer of this form is the rate-limiting factor in the hydrolysis of EAAC.[8]

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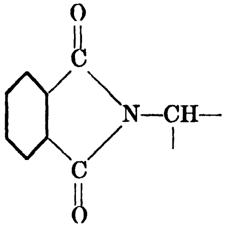
[6] Unpublished experiments. For preliminary data see (11).

[7] Note also the difference in proteolytic coefficients between GTA and BTA (Table I and Ref. 7).

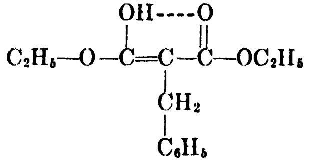
[8] If this were the prime factor responsible for the relative resistance of this compound to chymotryptic hydrolysis, it would follow that, contrary to previously advanced ideas (31), the specific peptide substrates of proteolytic enzymes do not enolize preparatory to enzymatic hydrolysis.

3. If *both hydrogens* of the α amino group of phenylalanine methyl ester are substituted as in phthalylphenylalanine methyl ester (PhPME Compound 20 of Table I), the compound is entirely resistant to chymotryptic hydrolysis. This compound not only lacks a hydrogen donor for hydrogen bond formation, but, in addition, free rotation of the residue beyond the nitrogen has been sacrificed by ring closure.



Both factors may contribute to the complete resistance of this compound to chymotryptic hydrolysis.

4. If the secondary peptide bond is replace by an *ester group,* as in benzyl diethylmalonate (BDEM, Compound 18 of Table I), the compound is very slowly hydrolyzed by chymotrypsin, if at all. None of the resonating structures (32) of the keto form of this compound possesses a hydrogen donating group, whereas the equilibrium concentration of enol form, which does have a potential hydrogen donor, is shifted too far toward the former to be measured experimentally (33).[9]



$$C_2H_5{-}O{-}\overset{OH\cdots}{C}{=}\underset{\underset{C_6H_5}{|}}{\underset{CH_2}{|}}{C}{-}\overset{\cdots O}{\overset{\|}{C}}{-}OC_2H_5$$

Moreover, internal hydrogen bonding in the enol form will reduce the hydrogen donating capacity of the compound toward the enzyme.

5. Replacement of the secondary peptide bond by a *carboxylate* group yields a compound, ethyl-*dl*-α-benzylmalonate (Compound 19 of Table I), which, of necessity, is devoid of the structural Component 1 of the parent compound. Its resistance to enzymatic hydrolysis in pH regions in which the carboxyl group is ionized may, therefore, be

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[9] These equilibrium measurements were carried out on diethyl malonate (33) but presumably apply also to the compound herein under discussion (BDEM).

ascribed to the lack of both a hydrogen donating group and the structural component beyond the secondary carbonyl group.[10]

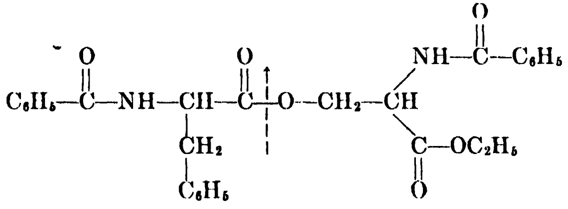
Component 3 (Hydrogen on α Carbon Atom)

Omission of the hydrogen on the α carbon atom yields unsaturated compounds of the type already described (EAAC), in which unsaturation may be the prime contributing factor.[11]

Component 4 (Susceptible Bond)

It has already been shown that the susceptible peptide bond of specific peptide substrates for chymotrypsin may be replaced by an amide or ester bond (7).

It has been suggested that, in proteins, internal ester bonds are formed between aliphatic hydroxyl groups and the secondary carboxyl group of dicarboxylic amino acid residues (34). To test this hypothesis on a model substrate which contains a typical amino acid residue for chymotrypsin, the ester formed between the hydroxyl group of serine and the α carboxyl group of phenylalanine was prepared, with both α amino groups masked by benzoylation, and the free carboxyl group esterified (N-benzoyl-DL-phenylalanyl-O-(N-benzoyl-DL-serine ethyl ester), BPBSEE).



BPBSEE

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[10] Measurements of the influence of temperature on K_m of the hydrolysis of specific peptide and ester substrates (BTA and BTEE) by chymotrypsin, yielded an average of $\Delta H = 9{,}000$ cal./mole (7). This value is compatible with the assumption that 2 hydrogen bonds per mole of substrate are formed in enzyme-substrate combination, one of them resulting from hydrogen donation by the imino group of the secondary peptide group, as postulated herein, the other one by hydrogen acceptance by the adjacent carbonyl group.

[11] Tests on substrates in which the hydrogen on the α carbon atom is replaced by another grouping would be conclusive only if the resulting compound is hydrolyzed by chymotrypsin. Resistance to enzymatic hydrolysis may have to be ascribed to steric hindrance by the substituent of larger size than hydrogen.

This compound was hydrolyzed by chymotrypsin very slowly, if at all, any measurable degree of hydrolysis having occured at the internal ester group, since benzoyl-DL-serine ethyl ester itself was entirely resistant to hydrolysis. Although in specific ester substrates for carboxypeptidase (5) and trypsin (35) the size of the ester radical does not seem to be a limiting factor in enzymatic hydrolysis, it is likely that the relative resistance of the present considerably more complex ester, BPBSEE, to chymotryptic hydrolysis is due to the steric influence of the larger and electronically more reactive seryl residue beyond the susceptible ester group.

Component 5 (α Methylene Group)

Elimination of the methylene group from residue R, yielding ethyl-DL-α-benzaminophenylacetate (EBAPA, Compound 14 of Table I) practically eliminates the enzymatic hydrolysis of the resulting compound.

Component 6 (Phenyl Ring)

The present data show that substitution of tyrosyl residue[12] by the tryptophyl residue (Compounds 4 and 9 of Table I) decreases the activation rate by one order of magnitude. The proportionate decrease in K_m renders the maximum proteolytic coefficients of the two substrates almost identical. The change in K_m and k' is probably induced by steric interference of the bulkier indole nucleus with activated adsorption on the enzyme surface.

Methionine is among the first amino acids which appear in pancreatic digests of proteins (36). It was to be expected, therefore, that either trypsin or chymotrypsin will hydrolyze methionyl peptides (or esters). Indeed, chymotrypsin hydrolyzes benzoyl-DL-methionine ethyl ester[13] (Compound 8 of Table I), albeit considerably more slowly than the corresponding phenylalanyl or tyrosyl substrates. This cross-reactivity of methionyl and phenylalanyl substrates is in accord with the biological (37–39) and structural (40) interrelation between a divalent sulfur atom and an allyl group on one hand, and the resemblance in chemical reactivity of allyl and benzyl derivatives on the other. If these substitutions are successively applied to the present methionyl ester, a compound results in which the benzene nucleus is one methylene group more distant from the α carbon atom than in the phenylalanyl ester.[14] This factor may contribute significantly to the lower susceptibility of the methionyl ester to chymotryptic hydrolysis.

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[12] Comparison should be more properly made with the corresponding phenylalanyl ester since the phenolic group enhances the susceptibility of the substrate to chymotryptic hydrolysis (*vide infra*). Such a comparison can be made here only indirectly by comparing ATrEE with ATEE, and BTEE with BPEE.

[13] Control experiments on the hydrolysis of BMEE by trypsin were entirely negative.

The resistance of threonyl or seryl analogs of BPEE to chymotryptic hydrolysis (Compounds 10 and 11 of Table I) is to be anticipated, since these residues fail to bear any direct or indirect relation to aromatic structures.

The higher rate of activation of N-benzoyl-*o*-nitro-L-tyrosine ethyl ester (Compound 6 of Table I), as compared to the tyrosyl analog, must be related to the influence of the electron-attracting nitro group on the phenyl ring to the activation process (41). From comparison with *o*-nitrophenol (pK = 7.5 (42)), it appears that the phenolic hydroxyl group of this ester is preponderantly ionized at the pH of the present measurements. The effect of the *o*-nitro group on the affinity for the enzyme is difficult to evaluate since the increased activation rate of BNTEE, as compared to BTEE, is paired with a corresponding increase of K_m.

The preceding considerations converge to the conclusion that the aromatic residue in position 6 serves as a point for attachment of the substrate to the enzyme, and, in addition, affects the ease of hydrolysis of the susceptible peptide, amide or ester bond in position 4, presumably by a positive inductive effect.[15] From comparison of the dissociation constants, K', of homologous carboxylic acids, it was shown that, for every methylene group which must be traversed, the inductive effects of the phenyl group decreases log K' by about one-third (44). If this electronic effect were the only contributing property of the phenyl ring of specific substrates for chymotrypsin, ethyl-DL-α-benzaminophenylacetate, EBAPA (Compound 14 of Table I), in which the phenyl ring is closer by one methylene group to the susceptible ester bond than in the corresponding phenylalanyl analog, should be much more rapidly hydrolyzed than BPEE. The inverse relation actually observed indicates the importance of steric factors in combination between the phenyl ring with the active groupings on the enzyme, which are sufficiently critical to be affected by a change in chain length by 1–2 Å. U. These conclusions are in full accord with those derived from analogous studies on specific inhibitors for carboxypeptidase (45).

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[14] By the same reasoning it may be surmised that N-benzoylcysteineamide or its ester analog, as well as cysteyl peptides, should be susceptible to chymotryptic hydrolysis since, in the cysteyl residue, the divalent sulfur has the same spatial relation to the α carbon atom as has the phenyl ring in the phenylalanyl residue. Studies on the chymotryptic hydrolysis of these and S-alkylcysteyl esters are in progress.

[15] If the electronic mechanism of hydrolysis is assumed to be the same for enzymatic and non-enzymatic reactions, a positive center on the carbon atom of the susceptible bond would enhance hydrolysis (43).

Component 7 (Phenolic Group)

The contribution of this group to the susceptibility of substrates to chymotryptic hydrolysis can be evaluated from comparison of BTEE and BPEE (Components 3 and 7 of Table I). The electronegative constituent of the phenyl ring in BTEE has an accelerating effect on activation rates. Its effect on enzyme-substrate combination is evidenced by the fact that K_m of BTEE is lower than K_m of BPEE although the respective k' values are in inverse order.

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SUMMARY

A systematic study has been made on the contribution of structural elements of typical peptide and ester substrates for chymotrypsin to the susceptibility to enzymatic hydrolysis. Various segments of a typical substrate have been modified by replacement by structurally related or dissimilar groupings, and the resulting changes in enzyme-substrate dissociation constant, K_m, and specific activation rates, k', have been correlated with the effects of chemical modifications on the steric and electronic properties of the substrates. From such an analysis, structural requirements of specific substrates for chymotrypsin have been defined within relatively narrow limits. These include: *1.* a secondary peptide group, or in its place a group capable of hydrogen bond formation by hydrogen donation; *2.* an enzymatically susceptible peptide, amide or ester bond; *3.* a tyrosyl, phenylalanyl, tryptophyl or methionyl residue attached to the α carbon atom; and *4.* relatively stringent spatial relations of the phenyl group, or its structural analog, of the amino acid residue to the bond which is hydrolyzed.

The synthesis of a variety of substrate analogs, active and inactive, has been described.

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Some Further Experiments on Red Yeast Polyenes

It was reported, in collaboration with A. Sandoval (1), that when the red yeast, *Rhodotorula rubra,* is induced to mutate by ultraviolet light, the respective mutants obtained show substantial changes in their polyene content. These differences include both the carotenoid pigments and the colorless, strongly fluorescing compound, phytofluene. Thus, red, brownish-orange, pale orange, and colorless mutants were obtained. The latter albino mutants were free also of phytofluene.

In the experiments mentioned, the preparation of the mass cultures and the chromatographic resolution of the respective polyene mixtures was carried out as soon as possible after the respective strains were obtained. In contrast, the present contribution presents some data concerning such polyenes, which were extracted after the original red yeast strain, or a mutant, had been submitted to a monthly transfer on agar during the period of 2.5 years.

This re-investigation shows a degree of genetical instability of the mutants, and, indeed, of our original strain as demonstrated by the quantity of some polyenes found at the end of the transfer period (Table I). The outstanding feature observed was the substantial decrease in the phytofluene content. Control experiments carried out with the original strain proved that neither variation in the agar concentration nor in the intensity of the illumination during the growth of the mass cultures can be held responsible for this change. It should also be mentioned that none of 4 further mutants, which had not been analyzed in 1945, now shows a high phytofluene content (Table I).

It is an interesting question whether or not the decrease in the phytofluene accumulation during the long transfer period is linked to increased pigment production. This was observed in at least one instance.

TABLE I

The Phytofluene Content in Some Rhodotorula rubra Mutants before and after a 2.5 Year Period of Monthly Transfers on Agar

(The figures give mg. of phytofluene/100 g. of dehydrated yeast)

	Spring 1945	Summer 1948
Original (red)	0.63 and 0.75	0.25
Mutant IV (deep red)	1.2	0.43
Mutant II (brownish-orange)	0.75	0.42
Mutant V (colorless)	0	0
Mutant VIII (deep red)	Not determined	0.35
Mutant IX (red)	Not determined	0.17
Mutant X (very pale yellow)[a]	Not determined	0.08
Mutant XI (colorless)	Not determined	0

[a] This mutant is of particular interest since it contains almost as much phytofluene as the sum of the other polyenes.

EXPERIMENTAL

In general, techniques such as those described earlier were used. In the nutrient medium the agar concentration was varied for the original *Rhodotorula* strain, *viz.*, to be 15.0, 20.0, 50, and 100 g/l. medium. These cultures were grown for 20–25 days before harvest.

To estimate the individual polyenes by chromatography, in some cases $Ca(OH)_2$ was used instead of a calcium hydroxide-alumina column. However, the absence of phytofluene, or its presence in only very small quantities, was rechecked on a 4:1 calcium hydroxide-alumina mixture in each case. The lime columns were developed with petroleum ether containing 4% acetone, and the mixed columns with the same solvent containing 7% acetone.

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Lycopersin, A Pigment from *Fusarium lycopersici*

The mold, *Fusarium oxysporum*, produces a pigment which has been called a "natural indicator, "since it changes color from red to blue in acid and alkali (1). This behavior is similar to that of *Aurofusarin*, a pigment formed by *Fusarium culmorum* (2).

A similar acid-alkali sensitive pigment is produced by *Fusarium lycopersici* (3).

Fusarium lycopersici shows a corresponding increase in both fat and pigment production with increased concentrations of glucose when grown on a Raulin-Thom medium (4). If the inoculations are made from stock cultures kept on potato dextrose-agar into a 5% glucose Raulin-Thom medium, heavily pigmented mycelia are obtained. The pigment does not diffuse into the medium.

Upon extraction of the mycelium with chloroform, *Lycopersin* is obtained as shiny, bright red needles from pyridine. It does not have a sharp melting point, but progressively darkens from 250°C. until it decomposes at 305°C. Similarly to *Aurofusarin*, this pigment is insoluble in most of the usual organic solvents.

From microanalyses of *Lycopersin*, recrystallized 5 times from pyridine, the formula $C_{20}H_{15}O_8$ can be derived:

Anal. Calcd. for $C_{20}H_{15}O_8$: C, 62.66; H, 3.92; OCH_3, 16.2. Found: C, 62.67; H, 3.81 (average of 6 analyses); OCH_3, 15.5, 15.6.

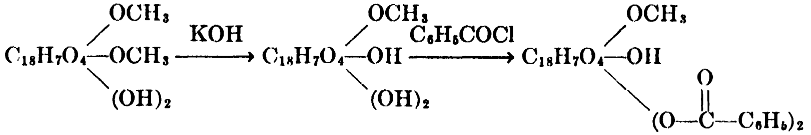
Since the dark color of the pigment and its insolubility prevented the determination of its molecular weight, a diacetate, dibenzoate, and di-*p*-nitrobenzoate were prepared. The analytical data obtained for these derivatives agree with the proposed formula:

Derivative	M.P. (°C)	Carbon		Hydrogen		OCH_3		$COCH_3$		N	
		calc. *per cent*	found *per cent*	calc. *per cent*	found *per cent*	calc. *per cent*	found *per cent*	calc. *per cent*	found *per cent*	calc. *per cent*	found *per cent*
Diacetate	281-2(d.)	61.67	61.62	4.07	4.31	13.3	13.2	18.4	18.7		
Dibenzoate	310(d.)	69.04	69.32	3.89	3.72	10.5	11.2				
Di-*p*-nitrobenzoate	280(d.)	59.91	59.85	3.08	3.39					4.11	4.48

Refluxing the pigment in aqueous KOH produced a deep violet solution. Methanol was identified in the alkaline distillate with chromotropic acid (5). Quantitative saponification showed that 4 moles of alkali were taken up per mole of pigment:

Wt. of sample	Meq. KOH	Meq./mole
0.2948	3.11	4.04

This saponified pigment was insoluble in 5% $NaHCO_3$ but formed a dibenzoate, and had lost only one OCH_3. Assuming that the resulting group was not benzoylated, the following reaction probably took place:



$$C_{18}H_7O_4\begin{cases}OCH_3\\OCH_3\\(OH)_2\end{cases} \xrightarrow{KOH} C_{18}H_7O_4\begin{cases}OCH_3\\OH\\(OH)_2\end{cases} \xrightarrow{C_6H_5COCl} C_{18}H_7O_4\begin{cases}OCH_3\\OH\\(O-\overset{O}{\overset{\|}{C}}-C_6H_5)_2\end{cases}$$

The microanalytical data agree with $C_{33}H_{21}O_{10}$:

Anal. Calcd. for $C_{33}H_{21}O_{10}$: C, 68.63; H, 3.64; OCH_3, 5.37. Found: C, 68.65; H, 3.76; OCH_3, 5.79.

In view of the influence of *Solanione* on the carbohydrate → fat conversion (4,6) in *Fusarium lini* B., the establishing of the structure of *Lycopersin* will be of particular interest.

This work is being carried on under the auspices of the Office of Naval Research.

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Book Reviews

Proteins and Life. By M. V. TRACEY, Rothamsted Experimental Station, Harpenden, Herts, England. The Pilot Press, Ltd., London, W. C. 1., England. 154 pp. Price 10s. 6d.

There is today an acute need for a short introduction to the fundamentals of protein chemistry, going more deeply into the subject than the average textbook of biochemistry, but avoiding the complexities of the advanced treatises on the subject. This little book by Dr. Tracey comes nearer than anything else I have seen to meeting this requirement.

He begins, after a short introduction, with a brief chapter on "What Proteins Are." This presents the standard classifications of proteins with due regard to the inadequacy of all methods of classification available today. This is followed by a very thoughtful discussion of the meaning of the term purity as applied to protein preparations, based largely on the earlier discussion by Pirie.

The second chapter, "What Proteins Are Made Of," presents the fundamentals of the chemistry of the amino acids, the hydrolysis of proteins, the methods available for the analysis of protein hydrolyzates, and some of the results obtained. Much of the more recent work in this field is incorporated here.

Chapter III on "The Structure of Proteins" makes use of numerous diagrams and protrayals of space models and polypeptide chains. The discussion is thoughtful and critical and, on the whole, sticks pretty closely to the established facts. A brief and qualitative discussion of methods of determining molecular weight is given, with several tables illustrating the sizes of proteins.

Chapter IV on "Proteins and Nutrition" begins with a discussion of the digestion of proteins and the specificity of some proteolytic enzymes, and continues with a discussion of essential and nonessential amino acids, protein requirements in nutrition, and some aspects of the intermediary metabolism of proteins.

Chapter V, "Sources of Proteins," gives an interesting portrayal of the nitrogen cycle in the light of modern knowledge.

Chapter VI, "Proteins in Industry," discusses the food indistry, animal protein fibers, proteins and plastics, and other related subjects.

Chapter VII, "Proteins and Life," discusses the ultra structure of a number of biological tissues, with special reference to their protein components.

Chapter VIII, "Proteins and Disease," gives a very brief presentation of some aspects of this subject.

It is easy to pick on some incidental flaws in a book of this sort, and every reader interested in the field would naturally wish to emphasize a somewhat different set of topics. I should have liked to see some further discussion of the acid and basic properties of amino acids and proteins, and a somewhat more quantitative treatment of the fundamental methods involved in the determination of melocular weight (the author scrupulously avoids mathematical equations—I do not think there is a single one in the book). I also should have preferred to see a larger portion of the book devoted to fundamental protein chemistry, and somewhat less space given to topics like nutrition and the industrial use of proteins. However, this book is certainly the best of its kind that I have seen, and indeed really stands in a class by itself as a simple introduction to the field. It can be warmly recommended to beginning students who wish to gain some perspective concerning the fundamentals of protein chemistry.

JOHN T. EDSALL, Boston, Massachusetts

The Chemistry of the Polysaccharides. By ROBERT J. McILROY, Senior Lecturer in Chemistry, Canterbury University College, New Zealand. Longmans, Green & Co., New York, N. Y., 1948. 118 pp. Price $2.50.

Although the title of the book is "The Chemistry of Polysaccharides," only about half of its contents (55 of a total of 118 pages) is devoted to this subject. The other half treats of a variety of subjects pertaining to carbohydrates including the following: Derivatives of the Monosaccharides, Estimation of Carbohydrates, Metabolism, Immuno-Polysaccharides, and an Appendix containing recent advances.

The author brought together in a small volume a considerable amount of valuable information, but unfortunately some of the statements are inaccurate or obsolete. This is especially pronounced in the chapter on the chemistry of starch. On p. 12, the periodate oxidation reaction which is the basis for a new method of end-group determination is erroneous. A starch chain consisting of glucose residues having a terminal non-reducing and a terminal reducing residue, will yield on periodate oxidation a total of 3 moles of formic acid (one from the non-reducing residue and two from the reducing residue) and not 2 moles as indicated by the author. The statement on p. 14 that the amylopectin starch component is insoluble, while the amylose component is soluble in water is also incorrect. Retrograded amylose is practically insoluble and amylopectin forms a colloidal solution in water.

On p. 20, under the discussion of "Enzymatic Synthesis of Amylose," the author states: "A most interesting development is the preparation of a synthetic starch from glucose-1-phosphate by C. S. Hanes." In this connection, no mention has been made of the amylose-like polysaccharide previously synthesized by Cori and Cori from glucose-1-phosphate by the action of muscle phosphorylase. Neither is this synthetic polysaccharide mentioned in the discussion of the chemistry of glycogen, p. 28. The assertion on p. 92 that: "Little is known about the mechanism of glycogen synthesis, but it appears that the glycolytic process is reversible," is not true. Actually there is more known about the mechanism of glycogen synthesis and its breakdown than about any other biochemical process.

The author has attempted to condense an enormous amount of material covering a variety of subjects into a booklet of 118 pp. Thus, animal carbohydrate metabolism is covered in 6 pages and plant metabolism in one page.

It is doubtful whether such a sketchy treatment could serve either the expert in the field or the student who merely wishes to get acquainted with the subject.

W. Z. HASSID, Berkeley, Calif.

The Fatty Acids and Their Derivatives. By A. W. Ralston, Assistant Director of Research, Armour and Co., Chicago, Ill. John Wiley and Sons, Inc., New York, N. Y., 1948. ix + 986 pages. Price $10.00.

The qualifications of the author deserve more than passing mention. Dr. Ralston is credited with more than 90 technical papers and 100 patents, as well as numerous other achievements in the field of fatty acid research. He was truly a pioneer in the commercial development and production of fatty acid derivatives. His activities as a director of research have given him a broad as well as detailed knowledge of the subject of which he writes. The reader senses that here is a compendium of information assembled by the author in the process of discovering for himself all that is known about the subject. In this book, he is passing on that fund of knowledge to present and future scientists, and in the author's own words "it will have served its purpose if it helps those who are to write that which is unwritten concerning this interesting group of organic compounds."

In keeping with the title, the subject matter is divided into two parts. The first part deals with the fatty acids themselves—their occurence in nature, their synthesis, and chemical and physical properties. In the latter department the author is not always critical, and frequently gives the reader a choice of physical constants without comment as to the probable accuracy. The second part of the book covers the synthesis, properties and uses of various fatty acid derivatives, including such diverse types as esters, amines, amides, nitriles, alcohols, ethers, mercaptans, sulfides, sulfonates, anhydrides, acid chlorides, aldehydes, ketones, hydrocarbons, soaps, and others. Much of this material has not previously appeared in book form.

There are many excellent features and a few minor weaknesses in the book. Organization of the subject matter is logical and the language is clear and accurate. The book is printed on good paper in easily readable type and is sturdily bound. Coverage of the literature up to about 1942 is exceptionally complete, with somewhat scattered references to later work. However, a digest of recent work included in an addendum serves to bring the book up to date. Admittedly this material would have been more useful if distributed throughout the text under appropriate headings. The book is provided with a 42 page topical index but lacks an author index. References to the literature, totaling more than 5,000, are listed in numerical order following each of the 12 chapters.

Despite the large number of references and the size of the book, errors are exceedingly rare. A few mistakes in nomenclature occur. For example, on p. 566, dicholesteryl adipate is listed as cholesteryl diadipate. In a few instances, alteration of the nomenclature used by original authors may introduce confusion.

All figures have been produced from original drawings and are of very high quality. Numerous tables add readability and facilitate location of data. However, there are many instances in which material that could have been tabulated has been run into the text.

Because of the breadth of the subject matter and the completeness of the coverage, this book will seve as a useful reference text, not only in fat and oil research, but in other fields in which the derivatives of fats and oils are utilized. It deserves a place in the libraries of practicing chemists as well as in the libraries of industrial and university laboratories.

A. W. Weitkamp, Whiting, Indiana

Fortschritte der Biochemie. 1938–1947. By Felix Haurowitz, Professor of Physiological Chemistry, Institute for Biochemistry and Medical Chemistry, University of Istanbul. S. Karger, Basel and New York, 1948. 365 + viii pp. Price 40 francs (Swiss).

The present volume is the fourth to be devoted to the "Progress of Biochemistry", the preceding volumes of the same title having appeared in 1925, 1931, and 1938, respectively. The longer period which has elapsed since the last presentation is, of course, related to the disturbances of the World War. In commenting on the new tools which have become available and which have contributed materially to advances in biochemical knowledge, Professor Haurowitz emphasizes the use of isotopes, the opening chapter being devoted to a general statement of the usefulness of the isotope procedures in the study of biochemical reactions.

The volume follows in general the usual scheme of presentation: minerals and their role in the living organism; the chemistry of the carbohydrates and related compounds; fats and lipides (in which group are included the carotenes, the sterols and sterides); protein chemistry and metabolism; heme, the porphyrins and derivatives; hormone proteins; vitamins and their functions; nucleic acids; hydrolytic enzymes; the chemical reactions of the nervous system and of muscular contraction.

More novel are a series of chapters devoted to various aspects of physical chemistry; *i.e.*, intermolecular forces in living materials, oxidation-reduction reactions, and the thermodynamics and kinetics of the living cells. The newer developments of immunochemistry, of antibiotics, and of cytochemistry are thoughtfully discussed. The final chapter is devoted to a presentation of newer analytical methods, useful in the study of biochemical problems.

The 24 chapters afford a review of modern developments of the more important aspects of biochemistry, although the greater emphasis has been placed on the vertebrates and on man. Although no subject is treated in monographic style, the discussions are carefully written and timely, with extensive bibliographic citations, including much of the work of 1947. They are more than statements of progress from 1938 to 1947, since, in order to present recent advances, it has been necessary in many cases to supply a background of historical development from the older literature.

One may well speculate on the extensive reading and careful study which must have been the preliminaries to the writing of this review. Few people in our own country would have been willing, or felt capable of undertaking such a task. Professor Haurowitz is to be congratulated upon successful completion of the volume. It can be placed in the hands of advanced students with confidence in its usefulness. Incidentally, those of us who are concerned in the training of graduate students will welcome a book in German which we can recommend to them not only as a means of improving their ability to read this language, but also for its content.

Howard B. Lewis, Ann Arbor, Michigan

Plant Viruses, 2nd ed. By Kenneth M. Smith, F. R. S., Virus Research Unit, Molteno Institute, Cambridge University. Methuen and Co., Ltd., London, 1948. ix + 78 pp. 8 plates. Price, 6 sh.

This book is a revised and rewritten version of the first edition, published in 1935. It is a member of the series of Methuen's Monographs on biological subjects, the purpose of which is to give authoritative though brief summaries of knowledge in various specialized fields of biology. These books are written for teachers and students of science, for investigators in other fields, and for the intelligent layman, but not for experts in the subject addressed.

Plant Viruses contains chapters on historical developments and the economic importance of viruses, on plant virus diseases, on modes of transmission of viruses, on viruses in insect vectors, on the chemical and physical properties of viruses, on serology and classification of plant viruses, on the control of plant virus diseases, and on the nature of viruses. Three of the 8 chapters are concerned primarily with the properties of plant viruses, and the remaining 5 are concerned primarily with the pathology and epidemiology of the diseases they produce. One hundred and two references to the literature and an overly brief subject index are appended.

The general style of the author is delightfully sophisticated. The reviewer enjoyed reading: "A question invariably put by the layman and by some scientists who should know better is whether viruses are living organisms. This is a question which cannot be answered, since there is no criterion of what is a living organism." The author was remarkably successful in maintaining balance, in spite of the condensed form which necessitated extensive omission.

Plant Viruses combines the virtues of readability, lucidity and authority. It is admirably adapted to the purpose for which it was intended. Apparently, it was written during the early months of 1946. The book should afford the general scientific reader an opportunity to grasp with a minimum of effort the most important contributions made prior to that time to plant virus research.

MAX A. LAUFFER, Pittsburgh, Pa.

Vacuum Manipulation of Volatile Compounds. By R. T. SANDERSON, Ph.D. John Wiley & Sons, Inc., New York, N. Y., 1948. viii + 162 pp. Price $3.00.

In the second paragraph of the preface to this book the author defines its scope as enabling the reader to ". . . construct and operate a general purpose high vacuum apparatus for chemical research." The title, however, refers to the manipulation of *volatile* compounds; and the title more accurately describes the contents. The much larger and expanding field of vacuum chemistry, which deals with chemicals of high molecular weight not ordinarily considered volatile, is ignored. The wide-necked still and molecular distillation are not mentioned, nor are the laws of gaseous flow or vapor evolution under high vacuum.

The author's main concern appears to be with vacuum techniques for transferring, measuring, and reacting small quantities—from mg. to ml.—of volatile carbon compounds, and reactive or spontaneously inflammable substances. The methods, with mercury seals, Töpler pumps, freezing traps, and stop cocks, are reminiscent of Ramsay and Travers and William Crookes; and of the Royal College of Science when the molecular weight of vanadium was being deterimned through the volatile chloride—was it in 1914?

A closer glimpse of the contents will reveal 15 pp. on elementary glass blowing, 2.5 on condensation pumps, and a 1.5 on the mechanical backing pump. "Measuring Pressure" occupies 5 pages, of which 2 are concerned with the McLeod gauge, and 2 with the mercury U-tube manometer. In contrast, there is a good chapter on freezing baths and low temperature measurements and an excellent appendix of vapor pressures, mostly of highly volatile substances. The Name Index reveals one reference each to such authorities as Dushman, Burch and Dunoyer, and three to Langmuir. Only one pioneer—A. Stock—draws more than 3 mentions with 12 references in various German journals, covering the years 1914 to 1922, and in one instance, 1932.

This little book will find acceptance, but less for its avowed subject matter than for the miscellany of early and unexpected information that the author has collected from the literature and his experience.

K. HICKMAN, Rochester, N. Y.

Fundamentals of Immunology, 2nd ed. By WILLIAM C. BOYD, Ph.D., Associate Professor of Biochemistry, Boston University, School of Medicine, Boston, Massachusetts. Interscience Publishers, Inc., New York. Interscience Publishers, Ltd., London, 1947. Price $5.50.

The second edition of Fundamentals of Immunology has been completely revised in accordance with new developments in the field. This edition reflects the progress made in studies of theoretical problems and also in the application of immunological methods. Considerable space in the new edition is devoted to the investigations concerning conjugated antigens, to the application of modern physical and chemical methods in the studies of immunological reactions, to the use of tracer substances, to the application of genetics' rule for immunological studies, *etc.*

Today, new methods of clinical and prophylactic medicine suggest a new consideration of immunological methods. Since the introduction of sulfa drugs and penicillin, the use of many immune sera has become obsolete, and Boyd has considerably shortened the section of his book dealing with such subjects as the properties of antigen for immunization purposes, the purification of sera, the technic of typing antipneumococcal sera, and others.

On the other hand, in connection with the increasing practice of blood transfusion, Boyd has extended the discussion of blood grouping and has written especially interesting pages on the application of this new science in forensic medicine. In this chapter as in all the others, a very extensive list of references is given, including most recent publications.

Another valuable feature of the new edition is that every chapter includes a very comprehensive summary. In addition, Table XLIII illustrates very clearly the uses and the limitations of immunological methods in preventing and treating infectious diseases. The chapter entitled "Laboratory and Clinical Technic "is an outstanding one. It should be valuable for reference to all who are engaged in the practical or experimental use of immunological technics.

The chapters on antigen and antibody reactions and on blood grouping are of particular interest, since the author has contributed so much to the knowledge of these subjects by his own research.

The book is written in a very clear style and, as a whole, offers a very objective presentation of every problem discussed. It should be stressed that, in many instances, it must have required a great deal of work and much skill to coordinate the conflicting information; for instance, in the field of the application of chemical and physical principles to the interpretation of immunological phenomena.

Boyd must be congratulated for his achievement in rewriting and revising Fundamentals of Immunology, which is an up-to-date book with abundant information, including very recent data and extensive lists of references. This edition can be extremely valuable as a text book for students, and also of great value to specialists and persons engaged in research in this field.

I. A. PARFENTJEV, New York, N. Y.

Cancer (Tome I), Hérédité, Hormones, Substances cancérigenes. By Dr. J. MAISIN, Professor, University of Louvain, Director of the Cancer Institute. Casterman. Tournais-Paris, 1948. 248 pp. Price: Francs 84.—

Dr. Maisin's book is designed for the educated general public. It is an up-to-date symposium of the fashionable cancer theories. Special emphasis is laid upon the relations existing between cancer and heredity, hormonal conditions in cancer, and chemical carcinogenesis. The book is written in the sparkling style customary with French authors.

HENRY K. WACHTEL, New York, N. Y.

A Symposium on the Use of Isotopes in Biology and Medicine. The University of Wisconsin Press, Madison, Wisconsin. 445 p. Price $5.00.

A desire to stimulate interest in the application of isotopes among those qualified to contribute to future advances prompted the Wisconsin Alumni Research Foundation to support a conference at Madison in September, 1947. The major part of the papers read at the Symposium is published in the present volume, which contains a great number of highly interesting contributions by competent authors. H. T. Clarke, who had the opportunity of witnessing at close quarters most important phases of the development of the application of isotopic tracers, gives, in the first contribution, an interesting survey of the historical background of the application of isotopes in biochemistry. This is followed by a paper by H. C. Urey on the separation of stable isotopes. The electrolytic separation of the hydrogen isotopes, the chemical separation methods, and thermal diffusion methods are discussed in detail, the conclusion being reached that, from the standpoint of cost of apparatus and operation, the most satisfactory method of separating the isotopes involved in biochemical problems is chemical separation. The detection of stable isotopes is the subject of a paper by A. O. Nier, who discusses, among other things, the merits of the highly sensitive null method of measuring isotopic ratios. The lucid presentation of the preparation of radioactive isotopes by G. T. Seaborg contains a statement, presumably unknown to many and certainly welcomed by all readers, that bombardment with 200 Mev deuterons can produce such hitherto unknown radioisotopes of biological interest as the 7.8 hr. positron-emitting Fe^{52} and the 9.5 hr. orbital electron-capturing Zn^{62}. The fabulous presentation of the Isotope Branch of the Atomic Energy Commission is vividly pictured by its Chief, P. C. Aebersold. We learn that, during the first year of distribution, more than 1100 shipments of pile-produced radioisotopes were made to 500 individual groups. C. D. Coryell gives, in 28 pages, a description of the fundamental principles of the detection and measurement of radioactivity. The great experience of M. D. Kamen in assaying carbon isotopes makes this author especially competent to describe the assay of radioactive carbon and other radioactive isotopes. Those interested in the preparation of labeled organic compounds will find valuable information in the contribution of D. B. Melville. Among the most beautiful chapters on the application of tracers belong the studies of the metabolism of proteins, which are reviewed by D. B. Sprinson, while H. G. Wood discusses among others the difficulties encountered in the presentation of the frequently ambiguous results of tracer studies on the intermediary metabolism of carbohydrates. The results obtained in tracer studies of the intermediary metabolism of lipides are reviewed by K. Bloch. The description of the application of radioactive indicators in studies on the nature of the defect in rickets and in numerous other problems on the metabolism of mineral elements, is the subject of a paper by D. M. Greenberg, who, together with his associates, has carried out many of the experiments described. The application of radioiodine in the study of the conversion of iodine into diiodotyrosine and thyroxine and the animal organism represents one of the most beautiful applications of radioactive indicators in biochemistry. This relationship is discussed in a detailed contribution of I. L. Chaikoff and A. Taurog, most of the remarkable results communicated being obtained by the Chaikoff school. The behavior of iodine in the human organism is discussed by a poineer in that field, J. G. Hamilton, who, in 17 pages, gives a survey of the medical application of radioactive tracers, including the interesting results obtained in the study of the distribution of fission products.

The next two interesting contributions, by B. E. Hall and S. Hertz, respectively, deal with the therapeutic use of radiophosphorus in blood diseases, and with that of radioiodine in thyroid disease. The advantages and disadvantages of radiophosphorus therapy in polycythemia vera are stated, the amount of radiophosphorus required to induce satisfactory emission being found to range between 5.0 and 35.0 millicuries. A review of the application of radioiodine in the clinical study and treatment of cases of hyperthyroidism and cancer of the thyroid is given, the risks and results of the treatment being likewise considered. The health hazards in the use of radioactive isotopes and the measures for the protection of personnel and property are discussed by W. F. Bale and J. J. Nickson, respectively. The closing contribution is an illuminating address by H. C. Urey on the international aspects of atomic energy. Those interested in the biochemical or medical application of rare or radioactive isotopes can be expected to find this volume delightful reading

G. Hevesy, Stockholm, Sweden

Erratum

In the article by Goranson which appeared in Volume 20, No. 1, pp. 15–21, the second paragraph of the Introduction should read as follows:

This demonstration of a "specific" inhibition of the primary esterification of glucose by monomeric glyceraldehyde *in vitro*, and the recent findings of Cori *et al.* (17, 18), suggested that an investigation be made of the action of the aldehyde administered to the intact animal with and without added insulin.

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The Physiological Activity of Some Simple Unsaturated Lactones. I. Effect on the Growth of Certain Microorganisms

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INTRODUCTION

Interest in certain physiological properties of some simple unsaturated lactones was stimulated when, in 1943, Medawar, Robinson and Robinson (15) synthesized DL-δ-hex-α-enolactone and showed that it pssessed growth-inhibitory properties similar to those of a lactone, $C_6H_8O_2$, isolated from malt extract. Kuhn and Jerchel (13) also synthesized the lactone and showed that its D-isomer was identical with "parasorbic acid" isolated from the fruits of *Sorbus aucuparia* by Hofmann (11), and whose constitution had been suggested by Doebner (6). A convenient synthesis, developed by Haynes and Jones (10) provided a ready source of unsaturated lactones, including hexenolactone, and enabled the present work to be undertaken.

When, in December, 1945, the synthetic lactones became available to us, published work concerning their physiological activity had dealt mainly with 3 aspects: (a) differential inhibition of the growth of animal tissues, (b) inhibition of the germination of seeds, (c) antibacterial activity. The last named had been confined to a study of the sensitivity of a limited number of bacteria and yeasts (14,2), and it was thought desirable to study the action of the lactones on other types of microorganisms, including types having different nutritional needs.

MATERIALS AND METHODS

The lactones used (kindly supplied by Dr. L. J. Haynes and Mr. M. C. Whiting) comprised the following:

DL-δ-Hex-α-enolactone (lactone of pent-1-en-4-ol-1-carboxylic acid; "hexenolactone").

DL-δ-Pent-α-enolactone (lactone of but-1-en-4-ol-1-carboxylic acid; "pentenolactone").

DL-γ-Hept-α-enolactone (lactone of hex-1-en-3-ol-1-carboxylic acid; "heptenolactone").

These were stored in 1% aqueous solution at 0°C.

The microorganisms, chosen to include nutritionally "non-exacting" and "exacting" types, were: *Fusarium caeruleum, F. fructigenum; Phycomyces Blakesleeanus, P. nitens; Nematospora (Ashbya) gossypii;* and *Lactobacillus arabinosus.* They were grown on the following media:

Fusarium species: Sucrose, 50 g.; KNO_3, 10 g.; KH_2PO_4, 5 g.; $MgSO_4,7H_2O$, 2.5 g.; $FeCl_3$, trace; water to 1000 ml. Inoculated from 7 day cultures grown on potato-agar slants; incubated at 25°C. for 10 days.

Phycomyces species: Glucose, 100 g.; KH_2PO_4, 1.5 g.; $MgSO_4,7H_2O$, 0.5 g. casein hydrolyzate (Ashe) 1.5 g.; thiamin, 50 γ; water to 1000 ml. Inoculated from 5 da cultures grown on malt-agar slants; incubated at 25°C. for 10 days.

TABLE I

Effect of DL-δ-Hexenolactone on the Growth of Various Microorganisms

Conc. of lactone in medium	Dry weight of mycelium, mg.				
	F. caeruleum	*F. fructigenum*	*P. Blakesleeanus*	*P. nitens*	*N. gossypii*
1 in 1,000	0	—	—	—	0
2,500	0 (3)	12–15 (3)	—	—	160–170 (14)
5,000	33–40 (15)	38–46 (15)	—	—	212–214 (14)
10,000	54–58 (3)	91–103 (3)	10–18 (6)	50–55 (10)	231–236 (14)
25,000	—	—	71–78 (8)	101–105 (10)	—
50,000	61–67 (3)	100–120 (3)	110–114 (10)	125–130 (10)	—
100,000	—	100–109 (3)	137–143 (10)	140–144 (10)	250–258 (14)
No lactone	66–78 (15)	148–156 (15)	165–175 (10)	170–176 (10)	260–270 (20)

(Figures in brackets show number of cultures used)

L. arabinosus

(The figures indicate ml. of 0.1 *N* NaOH required per tube)

Conc. of lactone	Concentration of Ça pantothenate, γ per tube				
	0	0.01	0.05	0.10	1.00
1 in 1,000	3.05	3.9	4.6	4.75	11.9
5,000	—	4.2	—	5.0	11.9
100,000	—	3.5	—	5.65	14.2
No lactone	3.15	3.9	4.8	6.4	14.5

Nematospora: Glucose, 20 g.; K_2HPO_4, 5 g.; $MgSO_4,7H_2O$, 2.5 g.; hydrolyzed peptone, equivalent to 3 g. nitrogen; *meso*-inositol, 0.1 g.; biotin, 50 γ; agar, 20 g.; water to 1000 ml. Inoculated from 5 day cultures grown on the same medium; incubated at 30°C. for 7 days.

Lactobacillus: The medium recommended by Barton-Wright (1) was used; incubated at 30°C. for 72 hr.

In all cases, double-strength media were made up, thus allowing for the addition of the necessary portion of the lactone solution (and other substances tested in conjunction therewith) together with water to give the final volume. Growth of the fungi was estimated from the dry-weight of mycelium produced; growth of *L. arabinosus* was assessed by alkali-titration of the acid produced during growth.

RESULTS

1. *Effect of Lactones on the Growth of the Microorganisms Tested*

Tables I and II show the effect of DL-δ-hexenolactone on the growth of all the microorganisms used, and of DL-δ-pentenolactone and DL-γ-heptenolactone on the growth of *Nematospora.*

TABLE II

Effect of DL-δ-*Pentenolactone and* DL-γ-*Heptenolactone on the Growth of Nematospora gossypii*

Conc. of lactone in medium	Dry weight of mycelium, mg. Pentenolactone		Dry weight of mycelium, mg. Heptenolactone	
1 in 1,000	91–98	(6)	35–43	(6)
5,000	117–177	(6)	173–185	(8)
10,000	217–222	(6)	187–195	(7)
100,000	237–240	(6)	217–225	(7)
No lactone	260–270 (20)			

(Figures in brackets show number of cultures used)

2. *Reversal of the Effect of the Lactones*

a. Experiments with Thiamine, Nicotinic Acid, Ascorbic Acid, Biotin, and Inositol. Since the growth of the fungi was usually markedly inhibited by the lactones when present at a concentration of 1 in 5000, this concentration was mostly used in experiments to study the possible reversal of the lactone effect by other substances. Attention was first directed to the use of some of the well-known growth factors in this connection. In view of the observation of Chakravarti (5) that the loss of germinative capacity of barley seeds exposed to ethylene oxide is parallel to the destruction of thiamine in the embryo, and the apparent similarity of this to the "blastocholine" property of hexenolactone (12), the possible protective action of thiamine received first attention. Tests showed that thiamine afforded no protection for these fungi. Similar tests were made with ascorbic acid, in view of its structural relationship to hexenolactone, and also because of the observation by Butler (3) that the bacteriostatic action of certain quinones on *Staphylococcus aureus* could be overcome by this vitamin. Further tests were made with nicotinic acid, and (for *Nematospora*) with biotin and inositol. In no case was any protective action found.

b. Experiments with α-Alanine, β-Alanine, Pantothenic Acid, and Pantoyl Lactone. Hauschka (7) and Hauschka, Toennis and Swain (8) demonstrated the partially protective action of α-alanine, β-alanine, cysteine, and glutathione against the characteristic lesions produced by hexenloactone in the case of the flatworm *Dugesia tigrina*. These workers used a system in which the effect of the lactone was to bring about disintegration of a tissue rather than to prevent a growth, but it seemed desirable to discover whether these simple compounds could afford protection against the lactones for the growth of susceptible microorganisms.

The two *Fusarium* species and *P. Blakesleeanus* were used. Lactone concentrations ranged from 1 in 5000 to 1 in 50,000, while β-alanine (*etc.*) was supplied in similar molar concentrations. Over a certain range of concentrations, β-alanine was found able to afford significant protection against the lactones. In each case there was an optimum concentration of β-alanine, further additions of which had less protective effect, although the amino acid itself was not toxic. In no case was the inhibitory effect of the lactone completely overcome, growth reaching only about 65% of normal in the most favorable cases. With *Phycomyces*, maximum protection was afforded when the molar concentration of the amino acid was about one-half that of the lactone, but no such relationship was apparent with other fungi (Tables III and IV). α-alanine was found to afford no protection against hexenolactone in the case of *F. fructigenum* (concentration of lactone, 1.7×10^{-3} *M*; concentration of α-alanine, 0.85×10^{-3} *M* to 3.4×10^{-3} *M*).

TABLE III

Effect of β-Alanine in Overcoming the Action of DL-*δ-Hexenolactone*

Conc. of β-alanine in medium	Dry weight of mycelium, mg.			
	F. caeruleum	*F. fructigenum*	*P. Blakesleeanus*	
	Lactone, 1.7×10^{-3} *M*	1.7×10^{-3} *M*	0.85×10^{-3} *M*	0.17×10^{-3} *M*
3.4×10^{-3} *M*	51–60 (10)	55.64 (10)	—	—
1.7×10^{-3} *M*	46–54 (10)	71–78 (10)	—	—
0.85×10^{-3} *M*	46–54 (10)	61–69 (10)	—	106–112 (4)
0.6×10^{-3} *M*	—	—	111–114 (4)	124–132 (4)
0.2×10^{-3} *M*	—	—	44–47 (4)	110–121 (4)
0	33–40 (10)	38–46 (10)	10–18 (3)	104–113 (4)
1.7 plus cysteine, 1.7×10^{-3} *M*	—	70–84	—	—
0; no lactone	66–78 (18)	147–157 (15)	129–145 (4)	

(Figures in brackets show number of cultures used)

Since β-alanine is probably an intermediate in the biosynthesis of pantothenic acid, this growth factor was also tested for protective action, but found to be entirely without influence in the case of *P. Blakesleeanus* and the two *Fusaria*. Pantoyl lactone was likewise without effect for *P. Blakesleeanus*, on which, tested alone, it had neither stimulatory nor inhibitory action.

The ability of cysteine partially to overcome the inhibitory effect of hexenolactone for *D. tigrina* was established by Hauschka (7). It seemed reasonable that the protective action of cysteine and of β-alanine, respectively, might be concerned with different steps in the metabolism, and that the two amino acids together might afford greater, or even complete, protection. Experiments showed that the effect of the two in combination was not significantly greater than that of β-alanine alone (Table III).

c. Miscellaneous Experiments. Butler (3) recorded the ability of ascorbic acid to overcome the bacteriostatic effect of certain quinones of *Staph. aureus;* tests showed that hexenolactone was without effect under similar conditions.

TABLE IV

Effect of β-Alanine in Overcoming the Action of *DL-δ-Pentenolactone and DL-γ-Heptenolactone*

	F. fructigenum; dry weight of mycelium, mg.	
Conc. of β-alanine	Pentenolactone, 0.9×10^{-3} *M*	Heptenolactone, 0.9×10^{-3} *M*
0.6×10^{-3} *M*	43–50	60–67
0.3×10^{-3} *M*	80–84	110–121
0.2×10^{-3} *M*	79–87	99–115
0.1×10^{-3} *M*	61–69	75–79
0	17–22	60–67
0; no lactone	142–147	140–147

(4 cultures used in each case)

Cavallito and Haskell (4) have demonstrated by simple physicochemical means that cysteine and some unsaturated lactones readily combine under "physiological" conditions, and suggest that such a combination acounts for the protective action of cysteine and also forms the pattern of the reaction which takes place between the lactone and some reactive compounds (*e. g.*, SH-enzymes) within the cell. Attempts to detect a similar direct condensation between hexenolactone and β-alanine were fruitless.

Discussion

The present work has extended the range of microorganisms known to be responsive to the growth-inhibitory action of simple unsaturated lactones, particularly DL-δ-hexenolactone. It has been shown that the growth of types of fungi differing widely in their nutritional requirements (*i.e.*, in their capacity for synthesis) is inhibited by the lactones in concentrations similar to those which are effective with animal tissues and bacteria. All the evidence obtained indicates that the lactone effect is not concerned with the metabolism of thiamine, ascorbic acid, nicotinic acid, biotin or inositol (for the microorganisms tested). That the lactones are not concerned with thiamine is further supported by an observation of Hardwick (private communication) that the carboxylase system of brewers' yeast is not affected by hexenolactone.

There would seem to be two groups of substances whose influence in counteracting the lactone effect is especially noteworthy, namely, certain sulphydryl compounds, and certain amino acids. The former have been considered to immobilize the lactones by forming simple addition compounds, thereby preventing the lactones from forming analogous compounds with functional SH groups of enzymes within the cell, but, as pointed out by Haynes (9), this simple theory is unlikely to give a complete explanation of the growth-inhibitory effect shown by so many unsaturated lactones (8,4).

Among amino compounds which exert a protective influence against the action of unsaturated lactones, the alanines seem to occupy an important position (7), but, in the present work, repeated attempts have failed to show any reversal of inhibition by α-alanine although the β-isomer was effective in every case examined. The fact that pantothenate is not antagonistic to hexenolactone strongly suggests that β-alanine has some function other than serving as a component of the pantothenate molecule. *Lactobacillus arabinosus* differs from the other microorganisms studied in being relatively insensitive to hexenolactones although at high concentrations of the lactone (in the presence of adequate pantothenate), growth is somewhat less. It differs from them also in needing an external supply of pantothenate, and, therefore, so far as the effect of the lactone is to interfere with pantothenate synthesis, its effect would not be apparent with *L. arabinosus*.

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We wish to thank Dr. L. J. Haynes and Mr. M. C. Whiting for supplying the synthetic lactones used, and Dr. F. Bergel for a gift of pantoyl lactone.

SUMMARY

1. DL-δ-Hex-α-enolactone inhibits the growth of *Fusarium caeruleum, F. fructigenum, Phycomyces Blakesleeanus, P. nitens, and Nematospora gossypii;* inhibition is complete at concentrations of 1 in 1000 of the

lactone, and marked at concentrations from 1 in 10,000 to 1 in 100,000. Growth of *Lactobacillus arabinosus* is only slightly inhibited.

2. Growth of *N. gossypii* is inhibited by DL-δ-pent-α-enolactone and DL-γ-hept-α-enolactone, but in neither case is inhibition complete at a concentration of 1 in 1000, although observable at 1 in 100,000.

3. In no case has it been found possible to overcome the inhibition by the addition of thiamine, nicotinic acid, ascorbic acid, biotin, or inositol, whether the organism needed an external supply of these substances or not.

4. In the case of *Fusarium* and *Phycomyces*, inhibition was partly overcome by β-alanine, growth then reaching an optimum of 65–75% of the normal. This protective influence was not increased by the further presence of cysteine, nor was it shown by α-alanine, pantothenic acid, or pantoyl lactone.

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The Oxidative Formation of Succinate in Higher Plants [1]

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INTRODUCTION

In an earlier study of the mechanisms of pyruvate metabolism in barley roots (5) it was suggested that the oxidation of pyruvic acid proceeded through a tricarboxylic acid cycle similar to that described by Krebs and Johnson (3). Bonner and Wildman (2), on the basis of respiratory studies carried out with spinach leaves, postulated a similar mechanism for the oxidation of pyruvate in the latter tissue. Since the strongest single criterion for the existence of a tricarboxylic acid cycle hinges upon the oxidative formation of succinate from fumarate, or from certain other dicarboxylic acids, the demonstration of this phenomenon was undertaken for both barley roots and spinach leaves.

MATERIALS AND METHODS

Accumulation studies with spinach were carried out with either fresh greenhouse spinach or bagged market spinach. Leaves were cut into pieces several cm. square, and approximately 25 g. fresh weight of this material was suspended in liter flasks containing 400 ml. of 0.05 *M* phosphate buffer, or buffer together with inhibitors, substrates, or both. The flasks were placed in a constant temperature (30°C.) bath, and the solutions aerated by means of sintered glass aerators which extended to the base of each flask. Manometric studies were carried out separately as described by Bonner and Wildman (2). The rates of oxygen uptake presented are for the third hr. of the experimental period. Microbial contamination appeared to be insignificant during the experimental period since the observed rates of gas uptake either remained constant, or diminished with time.

The barley employed in accumulation studies was of the variety Sacramento. Barley was germinated and grown as described by Ulrich (7), with the exception that 0.0025 *M* $CaSO_4$ solution was used during the growing period instead of a more elaborate nutrient solution. Five day old barley roots were excised at the seed, washed

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with distilled water, blotted between filter papers, and placed in phosphate buffer, or buffer together with malonate (pH 5.0), for a preliminary period of 1.5 hr. Thereafter the roots (approximately 15 g. fresh weight) were transferred to 100 ml. of experimental solution (Table VII) contained in macrorespirometers such as depicted in Fig. 1.

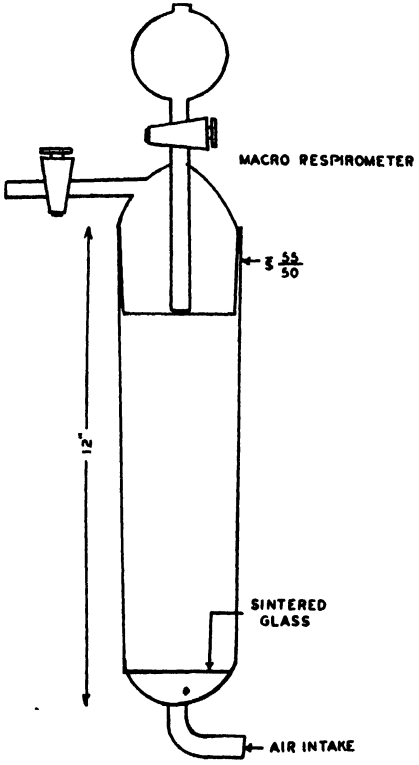

FIG. 1. Respirometer for large scale respiration studies.

The CO_2 arising from barley root respiration was continuously swept from the respirometer chamber by a stream of CO_2-free air, and collected in 50 ml. of 0.1 *N* NaOH contained in Pettenkoffer tubes 2 ft. long and ½ in. in diameter. At the end of the experimental period, 5 ml. of 10% $BaCl_2$, neutral to phenolphthalein, was added to 20 ml. aliquots of the alkali, and a double titration for carbonate was carried out with 0.1 *N* HCl, to the end points of phenolphthalein and methyl orange successively.

Succinate Determination

Tissue was dried in a forced draft oven at 70°C. (spinach) or under vacuum at 40°C. (barley roots). Thereafter, the tissue was powdered, acidified, extracted with ether, and the extract oxidized with permanganate, as described by Pucher and Vickery (6).

Succinate was then determined manometrically with the use of a succino-oxidase preparation obtained from sheep heart (8).

EXPERIMENTAL

Preliminary respiratory experiments were carried out to establish the concentrations of inhibitor and substrate most conducive to succinate accumulation. It was assumed that such accumulation would be most likely under conditions involving maximum inhibition of respiration by malonate, and maximum reversal of such inhibition by one or more substrates. Concentrations are expressed in terms of mg. of acid/ml.

TABLE I

Inhibition of Spinach Leaf Respiration as a Function of Malonate Concentration

Greenhouse spinach; pH 4.5

Time	Per cent inhibition of oxygen uptake			
	5 mg./ml.	10 mg./ml.	15 mg./ml.	20 mg./ml.
3 hr.	49	67	72	77
6 hr.	64	79	81	79

Although more rapid inhibition of oxygen uptake was achieved with increasing malonate concentrations up to 20 mg./ml., the final inhibition achieved by all concentrations in excess of 5 mg./ml., was approximately the same (Table I). Accumulation experiments indicated 5 mg./ml. to be the most satisfactory malonate concentration since tissue suspended in more concentrated solutions ultimately became flaccid. The low accumulation values at higher malonate concentrations (Table II) may be attributed to actual injury to the tissue. The

TABLE II

Accumulation of Succinate as a Function of Malonate Concentration

Greenhouse spinach, pH 4.5, 6 hr. experimental period

Malonate concentration *mg./ml.*	Succinate *mg./g. dry wt.*
0	1.10
5	2.64
10	2.20
15	1.75
20	0.81

TABLE III

Accumulation of Succinate as a Function of Time

Bagged market spinach, pH 4.5, malonate concentration 5 mg./ml.

Accumulation period *hr.*	Succinate *mg./g. dry wt.*
Control (zero time)	0.99
2	2.74
4	3.20
6	3.06
8	2.90

accumulation of succinate as a function of time is presented in Table III. In the presence of 5 mg./ml. of malonate, 3 mg./ml. of pyruvate gave the greatest respiratory response in a series of pyruvate concentrations ranging from 1 mg./ml. to 10 mg./ml. Response to fumarate addition was approximately constant at concentrations of 1 mg./ml. and above, in a range of concentrations from 0.25 mg./ml. to 5 mg./ml.

In the presence of malonate, the endogenous supply of fumarate is normally limited. In the experiments of Table IV, the concentration of fumarate was varied in the presence of optimal concentrations of pyruvate and malonate. The data indicate a definite interaction of pyruvate and fumarate. Fumarate concentrations which elicit no respiratory response whatsoever (0.25 mg./ml.) cause an appreciable increase in oxygen uptake in the presence of pyruvate. Thus fumarate or a fumarate derivative is involved in the oxidation of pyruvate.

TABLE IV

Interaction of Pyruvate and Fumarate in Spinach Leaf Respiration

Greenhouse spinach, pH 4.5, malonate concentration 5 mg./ml.

Oxygen uptake in excess of malonate-inhibited system ($\text{mm.}^3\text{O}_2$/200 mg. fr. wt./hr.)

Expt.	Control	Pyruvate	Fumarate			Pyruvate (3.0 mg /ml.) +fumarate		
		mg./ml.	*mg./ml.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	*mg./ml.*
		3.0	0.25	2.5	5.0	0.25	2.5	5.0
I	82	7	—	29	—	—	58	71
II	81	19	−2	—	37	38	—	68

Succinate Accumulation

The ability of spinach leaves to accumulate succinate in response to additions of particular substrates varied with the source of the tissue.

TABLE V

The Effect of Pyruvate and Fumarate on Succinate Accumulation

pH 4.5, malonate concentration 5 mg./ml. Accumulation period 6 hr.

Expt.	Tissue	Succinate concentrations in mg./g. dry wt.				
		Control	Malonate	Malonate + pyruvate (2 mg./ml.)	Malonate + fumarate (1 mg./ml.)	Malonate + pyruvate (0.5 mg./ml.) + fumarate (0.5 mg./ml.)
I	Bagged mkt.	1.51	2.54	3.74	3.60	3.88
	Bagged mkt. starved 24 hr. in dark at 27°C.	1.41	1.93	2.14	2.63	2.44
II	Bagged mkt. starved 24 hr. in dark at 27°C.	1.31	2.36	2.88	3.06	—
III	Fresh greenhouse	0.92	2.76	2.60	—	—
IV	Greenhouse, starved in pots for 2.5 days at 27°C. in dark	0.61	2.30	2.00	—	—

TABLE VI

The Effect of Pyruvate and Fumarate on Succinate Accumulation in the Presence of Fluoride and Malonate

Greenhouse spinach, malonate concentration 5 mg./ml., fluoride concentration 0.1 mg./ml., 2 hr. accumulation period, pH 4.5

Succinate concentration (mg./g. dry wt.)

Expt.	Experimental procedure	Control	Malonate	Malonate + fluoride	Malonate + fluoride + pyruvate (2 mg./ml.)	Malonate + fluoride + fumarate (2 mg./ml.)	Malonate + fluoride + pyruvate (1 mg./ml.) + fumarate (1 mg./ml.)
I	Fluoride added at start of exptl. period	1.35	2.84	2.56	2.74	—	—
II	Fluoride added 1 hr. previous to exptl. period	1.52	—	1.38	1.88	1.56	1.98

Of necessity, bagged market spinach was used in earlier experiments, whereas fresh greenhouse spinach was employed when it became available. Table V indicates succinate to be present in spinach to the extent of 0.1% of the dry weight. The succinate level is in all cases raised in the presence of malonate.

Although market spinach, in this series of malonate inhibition experiments, responds to the addition of both pyruvate and fumarate in terms of succinate accumulation, greenhouse spinach does not. With regard to the latter, neither preliminary starvation of whole potted plants, nor the use of short (2 hr.) experimental periods, resulted in the greater accumulation of succinate in the presence of added substrate than in its absence. However, the ability of this same tissue to accumulate succinate in the presence of malonate and in response to additions of fumarate or pyruvate was brought out in experiments with fluoride-inhibited tissue.

TABLE VII

Accumulation of Succinate in Excised Barley Roots

Malonate 1 mg./ml., 0.01 *M* KH_2PO_4, pH 5.0; accumulation period 6 hr.

Expt.	Treatment	Respiration *M* CO_2/g. fr. wt./hr.	Respiration as per cent of malonate-inhibited system	Mg. succinate/ g. dry wt.
I	None	—	—	0.19
	Malonate	161	—	4.1
	Malonate+pyruvate (4 mg./ml.)	236	147	9.2
II	Malonate	114	—	0.87
	Malonate+fumarate (5 mg./ml.)	190	166	9.85
III	None	—	—	0.0
	Malonate	130	—	3.3
	Malonate+fumarate (1 mg./ml.)+pyruvate (4 mg./ml.)	178	137	7.9

When substrates and malonate were added concomitantly with fluoride, little accumulation of succinate above that noted with malonate and fluoride alone was observed. If, however, the tissue was first treated with fluoride for 1 hr., subsequent additions of pyruvate, fumarate, or both, substantially increased the succinate concentration above that observed in the presence of fluoride and malonate alone (Table VI).

In excised barley roots, the succinate concentration in the absence of malonate may be too low to measure. In each case, malonate raises the level of succinate to where it is easily demonstrable (Table VII). Additions of pyruvate, fumarate, or mixtures thereof, increase the succinate level beyond that observed with malonate alone. Such increases invariably accompany stimulated respiratory activity, as evidenced either by increased CO_2 evolution (Table VII), or by an increase in oxygen uptake (5). The reductive formation of succinate from fumarate thus seems unlikely.

DISCUSSION

Krebs and Eggleston (4) have quantitatively described the conditions accompanying the oxidative formation of succinate in the presence of malonate. The overall representation of the process in minced pigeon breast muscle is as follows:

$$\text{Fumarate} + \text{pyruvate} + 2O_2 \longrightarrow \text{Succinate} + 3CO_2 + H_2O$$

Where malonate inhibition is complete, the reductive formation of succinate from fumarate is precluded, and the above equation describes the net result of a tricarboxylic acid cycle.

In the experiments presented above, the addition of pyruvate alone frequently results in an increased accumulation of succinate. The oxidative formation of succinate under these circumstances may occur by the combination of pyruvate with 4-carbon dicarboxylic acids already existing in the cell. Alternatively, the 4-carbon acids may be formed from pyruvate by the addition of CO_2 in a manner described by Wood *et al.* (9). The oxidation of additional pyruvate may then proceed as suggested by Krebs and Eggleston. Experiments with excised barley roots and $C^{14}O_2$ (unpublished) have indicated that CO_2 is readily taken up and incorporated into the carboxyl groups of the 4-carbon dicarboxylic acids.

If the additional CO_2 released in the presence of pyruvate is related as a molar quantity to the additional succinate accumulated in the presence of pyruvate, the ratio is consistently greater than that expected from the above equation. This aberration from the theoretical would be expected if some oxidation of succinate occurred continuously as a result of incomplete inhibition by malonate. Since malonate is a competitive inhibitor, it is reasonable to expect that some succinate oxidation may occur in spite of the presence of malonate when the succinate concentration has become high enough. This possibility is borne out by the apparent existence of an upper limit of succinate accumulation in both spinach leaves and barley roots. That is, for a fixed concentration of malonate, neither an increase in the duration of the experimental period nor an increase in substrate concentrations results in additional succinate accumulation beyond the maximum values presented.

Whether or not the accumulation of succinate in response to given substrates can be demonstrated, appears to depend on the nutritional status of the tissue (1). When the tissues are high in organic substrate (fresh greenhouse tissue), neither a preliminary starvation period nor a short experimental period permits the demonstration of an effect of added substrate. If, however, the source of pyruvate is curtailed by addition of fluoride, the effect of added pyruvate or fumarate on the accumulation of succinate is then manifest.

SUMMARY

The accumulation of succinate in the presence of malonate occurs both in spinach leaves and in excised barley roots. This accumulation is enhanced by pyruvate, fumarate, or both, under conditions which indicate the interaction of these compounds, or their derivatives, to be a fundamental step in the oxidative formation of succinate.

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Production, Isolation and Antimicrobial, Notably Antituberculosis, Properties of Streptothricin VI[1]

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INTRODUCTION

The ability of various strains of *Streptomyces lavendulae* and of closely related organisms to produce more than one type of antibiotic has been definitely established. Some of these substances, such as lavendulin (1) are characterized by antimicrobial spectra which are very similar to that of streptothricin (2,3); others produce mixtures of different antibiotics, such as streptomycin and streptothricin (4), streptothricin and streptolin (5,6), streptothricin and antibiotic 136 (7). The antibiotic spectrum of streptothricin is highly characteristic, as compared to other antibiotics also produced by actinomycetes, and it is basic in nature, such as streptomycin or aureomycin. Among the specific properties of streptothricin, one must recognize its activity against various fungi and its lack of or limited activity against certain bacteria, notably *Bacillus mycoides, B. cereus, and Serratia marcescens* (2,3).

The *S. lavendulae* group of organisms is widely distributed in the soil and in other natural substrates. These organisms can be readily recognized by their production of a characteristic rose-to-lavender-colored aerial mycelium, and by certain cultural properties, notably formation of a soluble brown to black pigment on gelatin, potato plug, and other protein- and peptone-containing media. Strains of *S. lavendulae* can thus be readily recognized and differentiated from other members of the *Streptomyces* group.

S. lavendulae produces mutants, the mycelia of which may lose their lavender color and become white, although the organisms usually

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retain their cultural and morphological characteristics, as well as their ability to produce a given antibiotic.

EXPERIMENTAL

Antibiotic Preparations

This paper deals with the isolation of an antibiotic substance belonging to the streptothricin complex from a strain of *S. lavendulae* No. 3516. This preparation is the sixth in a series of antibiotics reported to have been isolated from different cultures of *S. lavendulae* or from closely related organisms. The order of their announcement is, first, streptothricin, then streptin, followed by lavendulin, antibiotic 136, and streptolin.[2] This preparation may, therefore, be tentatively designated as "streptothricin VI" or as "SVI." In an unpublished paper from a Japanese laboratory, reference was also made to an antibiotic designated as "streptolycin," which definitely belongs to the streptothricin group of compounds.

Isolation and Testing of S. lavendulae 3516

The culture No. 3516 was isolated from a sample of soil that had been previously treated with various sulfur compounds. When tested, by the cross-streak method, it showed marked inhibition of growth of a number of gram-positive, gram-negative, and acid-fast bacteria, as shown in Table I. Glucose-free nutrient agar was especially suitable for the production of SVI, the presence of the sugar resulting in a marked reduction in the antibiotic effect. Very little of the active substance was produced in synthetic media, such as glucose-asparagine. Streptomycin-resistant strains of *E. coli* were sensitive to the antibiotic. When grown on nutrient agar, 3516 exerted a marked

TABLE I

Inhibition of Different Bacteria by 3516, Using Agar Cross-Streak Method

Test organisms	Zone of inhibition in mm. Nutrient agar	Zone of inhibition in mm. Glucose nutrient agar
Escherichia coli W	21	3
Escherichia coli R[a]	18	5
Bacillus subtilis	23	8
Staphylococcus aureus	19	6
Bacillus mycoides	5	4
Mycobacterium avium	22	11
Mycobacterium ranae	19	12
Mycobacterium 607	24	15
Mycobacterium 607R	19	12

[a] Streptomycin-resistant.

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[2] The press also announced the isolation in a commercial laboratory of another antibiotic ("valicidin") which apparently belongs to this group of compounds.

inhibiting effect upon the original streptothricin-producing culture of *S. lavendulae* (No. 3440), but the reverse was not true; the latter was also more sensitive than 3516 to the streptomycin-producing *S. griseus*. These facts tend to point to differences in the nature of the antibiotic produced by the two strains of *S. lavendulae*.

When 3516 was plated out and the individual colonies were isolated and tested, two types of cultures were obtained, one forming lavender-colored spores, which were characteristic of the mother culture, and another producing white spores. The pigmented strain grew more quickly and produced the antibiotic earlier than the white strain, which gave a greater yield of the antibiotic.

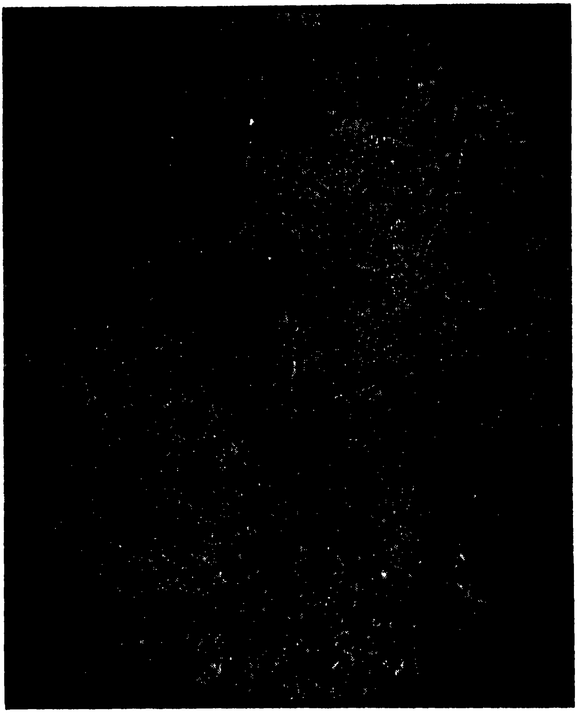

FIG. 1. *Streptomyces lavendulae* 3516.

Certain morphological differences were observed, however, between No. 3516 and the streptothricin-producing No. 3440, the former resembling more the original type species of *S. lavendulae* in the formation of spirals, whereas the latter does not form any spirals (Fig. 1).

Static cultures gave little surface growth, unless a solidifying substance was added, such as agar; no pellicle was produced in starch media. Without such a pellicle, very little of the antibiotic was formed. The culture was, therefore, grown in shaken cultures. Starch-tryptone broth gave a slightly higher activity than glucose-peptone-meat extract-NaCl media. The peak of activity was reached in 3 days. The pH at first decreased, then slowly increased to pH 8.55, and again decreased slightly.

Cultures of *S. lavendulae* 3516 were maintained on glucose-asparagine agar. Spores of 7 day old cultures were inoculated into flasks containing 100 ml. of the glucose-peptone-meat extract-NaCl broth; after 14–16 hr. on a rotary shaker, this culture inoculum was added to 500 ml. of the above medium in 2 l. baffle flasks. These flasks were then placed on a rotary shaker at 26°C. for 72 hr., when the highest antibiotic potency was obtained.

Methods of Concentrating Antibiotic SVI

Two methods were used for the removal of SVI from the medium and for its concentration, both of them being modifications of procedures used earlier in the isolation of streptothricin and streptomycin.

I. The culture medium was acidified to pH 2.0–2.5 with conc. HCl, stirred for 30 min. with Darco G-60 (5 g./l.), and Darco removed by filtration. The filtrate was adjusted to pH 7.0–7.3 with 40% NaOH, and stirred for 30 min. with Norit A (10 g./l.); the adsorbate was washed with 95% ethanol, and allowed to dry overnight at room temperature. The active substance was eluted, using the procedure of Peck *et al.* (8), yielding the formate of SVI. This preparation had a potency of 60–90 units/mg., a unit being equivalent in antibacterial potency to 1 γ of streptomycin, as determined by the cup or by the dilution methods. Recovery of 45–50% of original activity was thus obtained. The formate was further concentrated, by dissolving equal amounts of the preparation and of picric acid in water at 50°C., and finally transformed into the hydrochloride, with a potency of 200–220 units/mg., the yield being 45% of the formate.

II. The second procedure was a modification of the method of Bohonos *et al.* (7). The culture filtrate was acidified and treated as above. The neutralized filtrate was then stirred with Decalso, 1 g./8000 units of antibiotic, for 30 min. The adsorbate was filtered off, washed with water, and dried overnight in air. It was then placed in a column, 30 mm. inside diam., fitted with a plug of glass wool. A 10% aqueous NH_4Cl solution was allowed to percolate through the Decalso, and various fractions were collected and tested. The eluates having the highest potency were combined, adjusted to pH 7.0, and stirred with Darco G-60, 5 g./100 ml., for 30 min. The adsorbate was washed with water and dried, then treated with 0.05 *N* HCl in 50% methanol for 30 min. The filtrate was passed through a column of Amberlite IR-4B. The pH was thus raised to 6.0–6.5 without loss of activity or increase in salt concentration. The filtrate was concentrated *in vacuo* to remove the methanol, then filtered through a sintered glass funnel of "M" porosity, and lyophilized. The hydrochloride of SVI with a potency of 200–400 units/mg. was thus obtained from a broth which had an activity of 60–100 units/ml. The yield was 50–55%.

In another modification of this procedure, the charcoal eluate was concentrated *in vacuo* to dryness. The solid residue was fractionally dissolved in methanol and centrifuged. The antibiotic was precipitated from the supernatant liquid by addition of 10 vol. of acetone. By redissolving in methanol and reprecipitating with acetone, several preparations of different potency were obtained.

The SVI hydrochloride was further purified chromatographically by adsorption from a methanolic solution on acid-washed, activated alumina, at pH 4.5, with subsequent development and elution by methanol. Solids having a potency of 400–500 units/mg. were obtained by this procedure with 80% yield from solids having a potency of 200–300 units/mg.

Physical and Chemical Properties of SVI

SVI is stable at 5–25°C. at acid and neutral pH; there is no loss of activity under these conditions after 24 hr. An eluate from a decalso column showed no loss of antibiotic activity at 5°C. and at pH 6.5 over a period of 6 months. The loss of potency at 100°C. in 1 hr. at pH 2.0 was 10%; 50% of the activity was lost at pH 6.9 under these conditions. All the activity was lost at pH 10.5 in 40 min. at 100°C., 60% was lost in 1 hr. at 25°C., and 30% in 1 hr. at 5°C. The antibiotic is thus found to be most thermostable at pH 2.0, though it is relatively stable at icebox and room temperatures in acid and neutral solutions.

The passage of an aqueous solution of SVI through a sintered glass filter did not affect the potency, but the solution lost 40% of its potency when passed through a Mandler "W" filter and 96% when passed through a Seitz "EK" filter.

SVI is insoluble in ether, acetone, ethyl alcohol, and other organic solvents, except methanol. It is readily soluble in water. It is dialyzable. It is not inactivated by cysteine.

The specific rotation of the hydrochloride has been determined for solids of varying potency:

$[\alpha]_D^{25°}$ for solid having a potency of 200 units/mg. = $-$ 24.3° (c = 12.33 in water).

$[\alpha]_D^{25°}$ for solid having a potency of 500 units/mg. = $-$ 54.5° (c = 4.59 in water).

Antibacterial Properties of SVI

The antibiotic spectrum of the culture filtrate points to a similarity of this antibiotic to streptothricin, especially in its limited activity upon *B. mycoides* and *S. marcescens* and its considerable activity against fungi (Table II). In spite of the seeming similarity between the two antibiotics, certain quantitative differences were observed, which fully justified their further study.

On comparing the antimicrobial spectra of the two preparations with two other antibiotics produced by members of the *S. lavendulae* group

(Table III), a certain broad similarity was obtained, the differences being rather quantitative than qualitative. Other quantitative differences were obtained between the different antibiotics when other methods were employed. For example, when the cup method was used, streptothricin and SVI gave the same relative values as shown by the streak method; streptolin, however, gave only about one-quarter the activity, and antibiotic "V" only one-half the activity of streptothricin.

TABLE II

Comparative Antibiotic Spectra of Streptothricin and SVI

Units/ml. required to inhibit completely

Test organism	Streptothricin[a]	SVI
E. coli[b]	1.0	1.0
B. subtilis	0.2	0.1
B. mycoides	200.0	80.0
B. cereus	150.0	100.0
S. marcescens	125.0	30.0
S. aureus	3.0	5.0
Pr. vulgaris	1.0	1.0
Ps. fluorescens	70.0	30.0
M. avium	2.0	2.0
M. butyricum	3.0	4.0
Mycobacterium 607	0.7	0.8
Tr. mentagrophytes[c]	50.0	150.0
C. albicans[c]	300.0	300.0

[a] Both antibiotics were used in preparations obtained from cultures of corresponding organisms grown on glucose-peptone-meat-extract-NaCl medium.

[b] Both were active against streptomycin-resistant *E. coli* and *M. tuberculosis*.

[c] In some tests much higher potency was obtained than those reported here, namely, 5 units/ml. of the first fungus and 80 units/ml. for the second. They were also both active against *P. notatum*.

To emphasize further the quantitative differences in antibacterial behavior of the antibiotics produced by various strains of *S. lavendulae*, results of a series of tests made by the agar-streak method are presented in Table IV; for comparison, data obtained with a strain of *S. griseus* are also included. These results emphasize the qualitative similarity of the different *S. lavendulae* strains, as compared to the *S. griseus*. The differences in antibiotic behavior between the *S. lavendulae* strains are are definitely quantitative rather than qualitative.

When compared to streptomycin, SVI was found to repress completely all or nearly all of the cells in a sensitive bacterial culture and not to allow the development of resistant cultures. When a total of 9×10^{10} cells of *E. coli* were plated out on a series of 48 plates, no single resistant colony was observed on any of the plates. By growing, however, a sensitive culture in media containing increasing concentrations of SVI, resistant strains can be obtained.

TABLE III

Antimicrobial Properties (Antibiotic Spectrum) of Several Antibiotic Preparations Produced by Different Strains of Streptomyces lavendulae

Units/ml. of culture required for complete inhibition of test organism[a]

Test organism	Streptothricin	Antibiotic "V"	Streptolin	SVI
E. coli	1.0	2.0	0.8	1.0
S. marcescens	30.0	20.0	4.0	90.0
Ps. fluorescens	40.0	20.0	40.0	90.0
Pr. vulgaris	1.0	0.5	0.8	0.3
B. subtilis	0.2	0.2	0.4	0.3
B. mycoides	50.0	15.0	12.0	100.0
B. cereus	>50.0	>50.0	20.0	100.0
S. aureus	1.0	2.0	0.8	2.0
M. butyricum	0.5	0.5	0.2	1.0
M. avium	1.0	0.8	0.4	2.0
M. phlei	0.1	0.6	0.05	0.2
Mycobacterium 607	0.2	1.0	0.2	0.7
M. tuberculosis H37Rv	10.0	20.0	20.0	20.0
M. tuberculosis H37RvR[b]	20.0	—	600.0	20.0
Tr. mentagrophytes 171	20.0	15.0	10.0	>150
Tr. mentagrophytes 172	20.0	30.0	30.0	>150
C. albicans 173	70.0	40.0	4.0	>150

[a] The agar dilution streak method was used, except for the *M. tuberculosis* H37Rv and H37RvR, for which the turbidimetric method was used. Various antibiotic preparations contained the following streptomycin units/mg. of preparation, as determined by standard cup assay method: streptothricin—250, antibiotic "V"—200, streptolin—80, SVI—20.

It is to be recalled that the streptomycin unit was originally standardized against a streptothricin unit, namely, amount required to inhibit the growth of a standard strain of *E. coli* in 1 ml. of medium; 1 streptomycin unit later proved to be equivalent to 1 γ of streptomycin base.

[b] Resistant to streptomycin.

TABLE IV

Inhibition of Different Bacteria by Various Strains of Streptomyces by Agar-Streak Method

Nutrient agar used. Inhibition zone in mm.

Test organisms	*S. lavendulae* 3440[a]	*S. lavendulae* 3516[b]	*S. lavendulae* 3531[c]	*S. griseus* 3496[d]
E. coli W	27,28	25,24	23,23	19,18
E. coli R (streptomycin-resistant)	27,27	23,22	21,23	5,3
B. subtilis	31,31	30,30	25,26	24,26
B. mycoides 7a	8,10	5,4	6,5	20,23
Mycobacterium 607	29,27	28,29	28,25	28,26
Mycobacterium 607R (streptomycin-resistant)	24,24	27,27	25,19	22,17
M. avium	32,32	30,33	25,24	29,28
M. ranae 105	26,26	25,25	23,23	24,19
S. aureus	22,21	20,23	19,22	19,18

[a] Streptothricin-producing.
[b] SVI-producing.
[c] The antibiotic produced by this organism is also of the streptothricin type.
[d] Streptomycin-producing.

Tuberculostatic and Tuberculocidal Properties of SVI

Woodruff and Foster (9) first demonstrated that streptothricin is active against various mycobacteria. The saprophytic *Mycobacterium* 607 was inhibited by 1 unit/ml., but not by 0.6 unit/ml.; more than 1 unit/ml. was required to inhibit the avian strain. These workers did not, however, test the pathogenic strains of *M. tuberculosis*. Feldman and Hinshaw (10) tested the *in vivo* activity of streptothricin against *M. tuberculosis* and found it to be devoid of any important properties, as compared to streptomycin. Comparable results were obtained by Wagley and Steenken (11,12), who reported that even 100 units of streptothricin had only a temporary inhibiting effect upon the growth of *M. tuberculosis* H37Rv; after 28 days' incubation, this effect was no longer detected. Smith and Waksman (13) observed that although *M. tuberculosis* H37Rv was sensitive to streptothricin, it required 600 units of this antibiotic to inhibit the streptomycin-resistant strain H37RvR.

As pointed out above, SVI is almost as active against the resistant strain of *M. tuberculosis* as against the sensitive strain. A comparative

TABLE V

Bacteriostatic Effect of Streptomycin (SM) and of SVI upon Mycobacterium 607

Treatment	5 hr.	24 hr.	72 hr.
Streptomycin-sensitive 607			
Control	11,050,000	224,000,000	
SM 1 γ/ml.	0	<10,000	
SVI 1 unit/ml.	0	<10	
Streptomycin-resistant 607R[a]			
Control	653,000	12,300,000	19,000,000
SM 1 γ/ml.		5,180,000	10,000,000
SM 10 γ/ml.		2,190,000	8,000,000
SVI 1 unit/ml.	11,400		13,800,000
SVI 10 unit/ml.	0	0	0
SM 9 γ/ml.+ SVI 1 unit/ml.	7	<10,000	<100,000

[a] This strain was made resistant to 300 γ/ml. of streptomycin, as measured by the turbidimetric assay; the resistance was less when determined by the plate method.

TABLE VI

Effect of Different Concentrations of Streptomycin (SM) and of SVI upon Mycobacterium 607 as Determined by Agar-Plate Method

Treatment	24 hr.	72 hr.
	Streptomycin-sensitive 607	
Control	3,000,000	30,000,000
SM 0.1 γ/ml.		1,230,000
SM 0.2 γ/ml.		735,000
SM 0.3 γ/ml.		350,000
SVI 0.1 unit/ml.		1,740,000
SVI 0.2 unit/ml.		710,000
SVI 0.3 unit/ml.		300,000
	Streptomycin-resistant 607R	
Control	2,000,000	16,750,000
SM 10 γ/ml.	<10,000	1,925,000
SM 100 γ/ml.	<10	<10
SVI 5 unit/ml.	0	0

study was first made of the effect of streptomycin and SVI upon the sensitive and streptomycin-resistant strains of the saprophytic *Mycobacterium* 607, using glycerol nutrient agar. The resistant strain

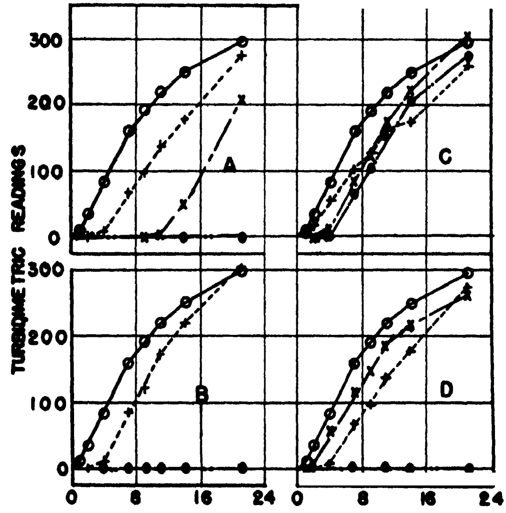

FIG. 2. Comparative effects of streptomycin (SM) and SVI upon the growth of *Mycobacterium* 607R in Dubos medium. A. Streptomycin series: ○○○○○○○ = control; —+——+—— = 5 u/ml.; +++++++ = 10 u/ml.; · · · · · · · = 400 u/ml. B. SVI series: ○○○○○○○ = control; —+——+—— = 1 u/ml.; · · · · · · · = 2 u/ml. C. Streptomycin + SVI: ○○○○○○○ = control; —+——+— = 0.5 u/ml. SM; +++++++ = 1 u/ml. SVI; · · · · · · · = 0.5 u/ml. SM + 1 u/ml. SVI. D. Streptomycin + SVI: ○○○○○○○ = control; —+——+— = 5 u/ml. SM; +++++++ = 1 u/ml. SVI; · · · · · · · = 5 u/ml. SM + 1 u/ml. SVI.

(607R) was obtained by serial passage of the sensitive organism in cultures containing streptomycin. Dubos Tween 80 medium was inoculated with the two strains and incubated at 37°C. After 5, 24, and 72 hr. incubation, the cultures were plated on the glycerol agar, containing different concentrations of each antibiotic alone or mixtures of streptomycin and of SVI. The plates were incubated for 96 hr. and the number of colonies counted. The results (Table V) confirm the previous observations of the effectiveness of SVI upon the streptomycin-resistant strains of *Mycobacteria* and point to the possibility of obtaining a synergistic effect of the two antibiotics. Streptomycin in concentration of 1γ/ml., one unit of SVI, or 0.9 γ/ml. streptomycin + 0.1 unit SVI was sufficient to inhibit completely the growth of *Mycobacterium* 607 after 5, 24, and 72 hr. incubation. *Mycobacterium* 607R showed resistance to 10 γ/ml. of streptomycin at all times and a rather high resistance to 1 unit of SVI. When the two antibiotics were combined in the ratio of 9 γ/ml. of streptomycin + 1 unit of SVI, there was nearly complete inhibition of growth of the resistant organism.

The last experiment was repeated with lower concentrations of the two antibiotics (Table VI). The bacteriostatic effect of SVI was definitely of the same order of magnitude as that of streptomycin, except SVI was also active upon the streptomycin-resistant strain of *Mycobacterium* 607. Further data concerning the comparative effects of the two antibiotics upon the resistant strain of this organism are given in Fig. 2.

TABLE VII

Synergistic Effect of Streptomycin and SVI on M. tuberculosis H37RvR

Dubos Tween 80 medium used

Concentration of antibiotic		Turbidimetric readings expressed in logs			
SM	SVI	7 days	14 days	21 days	35 days
γ/ml.	*units/ml.*				
0	0	52	120	167	242
20	0	5	28	122	204
5	0	54	128	138	261
0	0.5	86	162	206	300
0	5	62	136	190	274
20	0.1	32	45	108	230
20	0.5	0	0	50	162
20	1	0	0	0	0
20	5	0	0	0	0
10	0.1	0	0	61	201
10	0.5	0	0	0	0
10	1	0	0	0	0
5	0.1	0	0	60	193
5	0.5	0	0	0	0
5	1	0	0	0	0
2.5	0.1	34	79	96	181
2.5	0.5	0	0	0	0
2.5	1	0	0	0	0

A study was next made of the comparative effects of streptomycin and SVI against H37RvR, or the streptomycin-resistant strain of the pathogenic form of *M. tuberculosis* H37Rv, using liquid media and measuring growth turbidimetrically (Table VII). When the concentra-

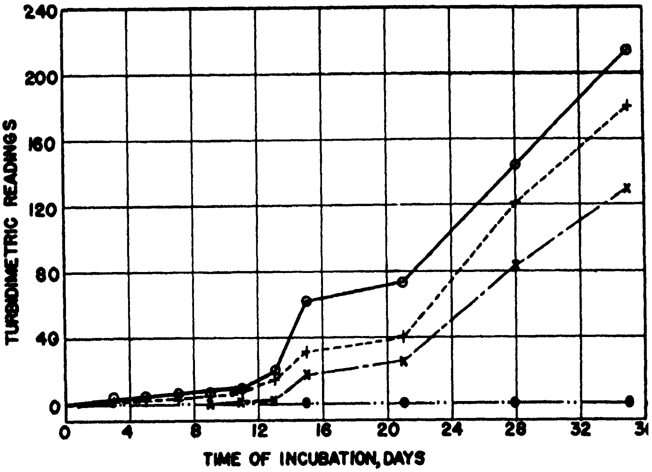

FIG. 3. Effect of SVI on the growth of *M. tuberculosis* H37Rv in Dubos medium. OOOOOOO = control; —+——+—— = 0.5 u/ml.; +++++++ = 1.0 u/ml.; · · · · · · · = 5.0 u/ml.

tion of SVI was 5, 1, or 0.5 unit/ml., and that of streptomycin was 20, 10, 5, or 2.5 γ/ml., there was complete inhibition of growth even after 35 days. Neither of the antibiotics alone gave any appreciable inhibition of the growth of the organisms in the above concentrations. This sug-

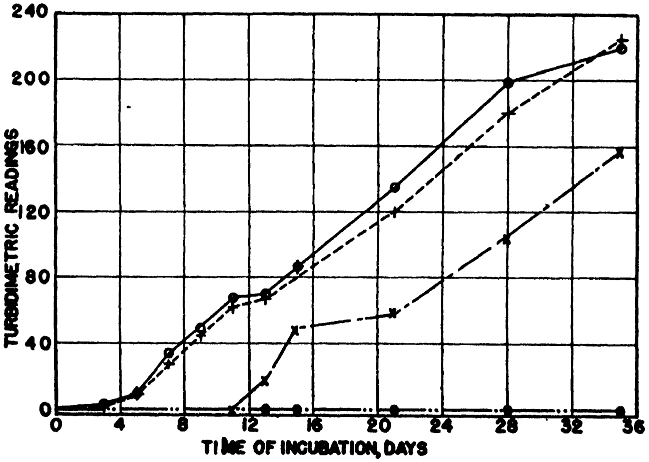

FIG. 4. Effect of SVI on the growth of *M. tuberculosis* H37RvR in Dubos medium. OOOOOOO = control; —+——+— = 1.0 u/ml.; +++++++ = 5.0 u/ml. · · · · · · · = 10.0 u/ml.

gests further a possible synergistic action of the two antibiotics, since more than 5000 γ/ml. of streptomycin is required to inhibit the growth of this strain of *M. tuberculosis*.

When the course of activity of SVI upon the human pathogen was measured by the turbidimetric method, complete inhibition of the H37Rv was obtained by the use of 5 units of SVI and that of H37RvR by 10 units, after 42 days' incubation, as brought out in Figs. 3 and 4.

Relative Tuberculostatic and Tuberculocidal Properties of Streptomycin and SVI

In a comparative study of the antituberculosis effect of the two antibiotics by the cup method, nutrient agar was used. The agar was seeded with a 24 hr. culture of *Mycobacterium* 607 from Dubos Tween 80 medium. Aqueous solutions of streptomycin (10 γ/ml.) and SVI (10 units/ml.) were placed in cups and plates and incubated. The zone of inhibition of growth was measured. After incubation at 37°C. for 40 hr. there were 2 distinct zones of inhibition for both antibiotics, an inner zone of complete inhibition of 23 mm. and an outer zone of 30 mm. Upon further incubation, for 92 hr., the zones produced by SVI remained as they were originally, whereas those produced by streptomycin were overgrown as a result of development of resistant colonies.

TABLE VIII

Resistance of E. coli to Varying Concentrations of Streptomycin and SVI

Numbers of cells in 24 hr. old culture of *E. coli* plated in nutrient agar containing varying concentrations of the two antibiotics.

Streptomycin, γ/ml. of agar	SVI, units/ml. of agar					
	0	0.5	1	2	3	4
0	335,000,000	244,000,000	10,000	1,250	322	35
0.5	309,000,000		5,900	890		
1	259,000,000	22,100	3,060	620		
2	45,000	2,230	1,170	107		
3	6,100					
4	1,790					

When an attempt was made to determine the potential synergistic effect of the two antibiotics upon this organism, using the cup method proposed by Robbins *et al.* (14), no enlarged zone was obtained; however, there were no resistant colonies of the organism in the zones where the effects of the two antibiotics were fused together.

To illustrate the development of resistant cultures against SVI, as compared to streptomycin, the results of another experiment are reported in Table VIII. There is a somewhat lower tendency for development of resistance to SVI than to streptomycin. This is carried over into the mixtures of the two antibiotics.

Comparison of the tuberculocidal properties of the two antibiotics, using a saline suspension of cells of 607, showed that streptomycin, however, exerts a far greater bactericidal effect than does SVI.

In Vivo Activity of SVI

SVI was tested for its *in vivo* potency and was found to have a relatively limited toxic effect upon animals. It protected animals against *Salmonella schottmülleri* as well as against the streptomycin-resistant strain of this organism. When large doses of the antibiotic were injected into mice (3000 units/20 g. mouse), delayed toxicity was obtained. Lower doses (50, 100, 250, 750 units/20 g. mouse) showed no toxic effects. This low toxicity of SVI was obtained only for preparations of the antibiotic which was isolated from cultures grown on the No. 3 medium. When 3516 was grown on the streptothricin medium (tryptone-starch), the resulting product also possessed the delayed toxicity characteristic of streptothricin.

ACKNOWLEDGMENT

The authors are indebted to Mr. Otto Graessle, of the Merck Institute for Medical Research, for determining the toxicity and *in vivo* activity of SVI. They also want to express their appreciation to Dr. D. M. Reynolds and to Mr. W. P. Iverson of this laboratory for assisting in the isolation of the culture and in making various determinations with SVI; to Mr. E. Katz, for assisting with some of the tuberculostatic and tuberculocidal studies; to Dr. W. H. Peterson of the University of Wisconsin for supplying a sample of streptolin and to Dr. A. R. Menotti of the Bristol Laboratories for supplying the preparation designated here as antibiotic "V".

SUMMARY

1. Members of the *Streptomyces lavendulae* group are widely distributed in nature, the variations in the cultural, physiological and biochemical properties of these organisms being rather quantitative than qualitative. Mutants are produced which may vary in one particular property, such as pigmentation of aerial mycelium.

2. Members of the *S. lavendulae* group produce a variety of antibiotics. The first antibiotic isolated from one of the cultures was streptothricin. This antibiotic, alone or in admixture with other antibiotics, is produced by a number of cultures of *S. lavendulae*. Other antibiotics, isolated from various cultures of this organism and designated as lavendulin, streptolin, *etc.*, are similar to streptothricin in many of their properties, notably nature of antibiotic spectrum.

3. A streptothricin-like material, designated as SVI was produced by a culture of *S. lavendulae* No. 3516, isolated from the soil.

4. Antibiotic SVI is similar to streptothricin in its chemical and antibacterial properties, but appears to be somewhat less toxic.

5. SVI possesses strong tuberculostatic and tuberculocidal properties against both the streptomycin-sensitive and the streptomycin-resistant strains of *M. tuberculosis.*

6. The possible synergistic action of SVI and streptomycin against *M. tuberculosis* is indicated.

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Content of Folic Acid in Normal and Diseased Human Hearts

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INTRODUCTION

The object of the present work is the determination of folic acid in human cardiac muscle before and after enzymatic digestion.

EXPERIMENTAL

Human hearts obtained immediately after autopsy were weighed and ground with 5 volumes of water in an electric blender. The resulting mass was placed in a hydraulic press and the fluid expressed. Folic acid was estimated in the liquid by the method of Teply and Elvehjem (1) using *S. faecalis*. Synthetic folic acid (courtesy of Ingram laboratories) was used as standard. This compound has the same potency as the naturally occurring material (Bc and RLR factor) for *S. faecalis* (2) and for animals. The apparent free folic acid in the cardiac muscle is shown in the table.

TABLE

Condition of the heart	No. of specimen studied	Apparent free folic acid in the myocardium	Folic acid after enzymatic digestion
	Values expressed as mγ/g., average		
Normal	3	0.85	38.00
Rheumatic endocarditis	4	0.83	36.80
Hemiplegia	2	0.80	31.10
Cardiac failure	2	0.89	38.90
Coronary insufficiency	2	0.79	30.90
Arterial hypertension	2	0.75	30.50
Luetic aortitis	2	0.78	30.00
Pancarditis	2	0.82	35.60

Folic acid was also determined after treatment with takadiastase. For this purpose the fresh weighed muscle (1 kg.) was triturated in a liquefier with 15 volumes of water and 150 g. of takadiastase. One liter of sodium acetate, 2.5 *M*, and sufficient 0.1 *N* HCl to bring the pH to 4.5 were added, and the mixture incubated for 16–18 hr. at 37°C., after with it was autoclaved for 15 min. to inactivate the enzyme. When cool, the mixture was filtered in a press, and aliquot parts were taken for analysis by the technique mentioned above; results are noted (see table).

CONCLUSION

Determination of free folic acid in human hearts revealed no difference between healthy and diseased hearts. The average concentration was 0.81 mγ/g. of muscle. After enzymatic digestion the concentration was increased to an average of 34.20 mγ/g. of muscle, again with no difference between normal and diseased hearts.

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The Biogenesis of the Anthocyanins. I. General Nutritional Conditions Leading to Anthocyanin Formation[1]

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INTRODUCTION

The anthocyanin pigments of plants have been subjected to very extensive chemical investigations. Through the classical isolations of Willstätter and his coworkers, and the brilliant synthetic work of Robertson and Robinson and of Robinson and Todd, the structures of virtually all these pigments in their naturally-occurring glycosidic forms are known.

Furthermore, in their role as flower pigments, these substances have been the subject of a series of genetical studies which provided the first analysis of the relations between genes and their products in chemical terms. Nevertheless, in spite of this interest and study from both the chemical and the biological side, the central area, namely the physiology of these substances, remains unknown. Neither their biogenesis nor their physiological function, if any, has been adequately studied. In this respect the anthocyanins are not alone, for the same is true, to varying extents, of most of the glucosides, the tannins, the essential oils and the alkaloids.

In the case of the anthocyanins, there is a particular reason for the lack of extensive physiological study. Their occurrence as flower pigments places them in a plant organ which is ephemeral in nature, which cannot be cultured in an isolated condition, and of which the basic physiology is little understood. For these reasons it has long been the authors' feeling that a successful study of anthocyanin development could only be initiated in green tissue. Leaves of *Coleus, Begonia,* corn, red cabbage, or of maple trees in the fall provide obvious possibilities. Some of these objects have, in fact, already been used in investigations of a physiological nature.

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[1] This work was made possible by a grant from the Milton Fund of Harvard University.

No attempt will be made here to review in detail the voluminous literature, since a very complete treatment has been given recently by Blank (2), while the earlier work was covered by Mrs. Onslow (15). The salient biochemical points which have been brought out by previous investigators, however, can be summarized briefly. Following the work of Ewart in 1897 (5), who immersed *Elodea* and Utricularia in sucrose solutions and found that they reddened, particularly in bright light, an extensive study was made by Overton (16). Overton showed that *Hydrocharis morsus-ranae* turned deep red in 0.5–3% sucrose, glucose, fructose, or invert sugar solutions. Lactose, galactose, glycerol, and inorganic salts had no effect. Light was essential for the reaction. *Elodea*, *Vallisneria* and some *Potamogeton* species behaved similarly, but other aquatics, including *Lemna minor* and *L. trisulca*, showed no effect. Lowered temperature, in general, promoted reddening. Some plants, whose leaves never show reddening, did not respond to sugar feeding. Extensive results, very similar to those of Overton, were obtained by Katic in 1905 (13), who found also in a few cases, notably in *Hydrilla verticillata*, that red pigment could be produced with sugar in the dark. This fact was confirmed by Gertz (7), although, in general, light certainly promotes pigmentation. In some plants light without sugar suffices to cause reddening. In one such case, that of buckwheat seedlings reddening on exposure to light, Karstens (12) found a reasonably close relation between the distribution of sugar in different zones of the hypocotyl and their ability to redden. However, histochemical detection of sugar in the epidermis failed, although these cells form plenty of pigment. The lower limits of the histochemical method are not known and the epidermis certainly receives the most light, so that this exception is not necessarily critical, and may be compared with reddening of aquatics in bright light without sugar. In this connection, the observation of Hicks (11) that *Spirodela* is redder in sunlight than in shade is pertinent.

All of the evidence thus agrees that there is a close relation between sugar content and anthocyanin formation. Recent work by Fogel and Stadler (largely unpublished) shows that corn leaves may be induced to form anthocyanin when floated on sugar solution; in this, glucose and sucrose were equally effective. Frey-Wyssling and Blank (6), with red cabbage seedlings (*Brassica oleracea* var. *capita rubra*), found that the relation between reducing sugar content and anthocyanin formation was only a general one and did not hold strictly for different ages and temperatures. Thus, other factors besides sugar must influence the formation of pigment, and, doubtless, under varying conditions different factors might become limiting. It is difficult either to draw reliable conclusions or to compare the results of different investigators for two reasons; firstly because actual anthocyanin content was never determined (except in the papers of Frey-Wyssling and Blank and of Karstens), and secondly because practically every worker has used different material. It is clear that both a quantitative method for pigment assay and a readily cultured, reproducible object are essential for more detailed investigation.

A particularly favorable type of experimental material was disclosed by one of us in the course of an investigation on bound auxin (21). Experiments were carried out on *Lemna minor*[2] because it could be cultured under constant conditions on a standard medium; the material was homogeneous and so thin that it did not need to be sliced; nevertheless, it is a flowering plant nad thus comparable with the larger forms. During these experiments, it was noticed that aqueous protein-free extracts, prepared from plants which had been grown for some days under high light intensity, were of a clear purple hue; and this suggested the use of the *Lemnaceae* for an investigation of anthocyanin physiology.

The Experimental Plant

The *Lemnaceae* have proved suitable for this work for the reasons given above as well as some supplementary ones: as higher plants they are comparable to other species in their general physiology; under experimental conditions they reproduce only vegetatively, however, so that genetic variation need not be considered. They can be freed from contaminants and grown in sterile culture with any desired organic substrate. Because the individual plants are small in size, and floating directly on the solution, problems of translocation do not arise. Finally, it has been possible to develop a simple and accurate method of pigment analysis based upon the observation mentioned above.

The species used in these studies in *Spirodela oligorrhiza* Kurtz. Anthocyanin is formed first at the margins of the fronds, and, when some organic pigment precursor is supplied in the medium, at the root base. It then appears in a reticular pattern on the underside of the fronds, presumably following vascular paths; and then it spreads over the rest of the ventral surface of the fronds. The rate of pigment formation is such that, while sufficient pigment for analytical purposes is produced in a reasonable time, experimental variations can affect the rate enough to be measurable. This is not true of *Spirodela polyrrhiza*, in which the rate of pigment formation is so great that large amounts appear whenever growth proceeds. The growth habit of *S. oligorrhiza* is also favorable in that the fronds separate from one another soon after they are formed, providing small plants for transfer purposes.

Spirodela is separated from *Lemna* mainly on the basis of its possession of more than one root to each root base. Since these plants rarely flower, the morphology of the flowering parts is of less taxonomic significance than in most higher plants; in several of the described species flowers are unknown. In almost every character, *Spirodela oligorrhiza* lies midway between *Spirodela polyrrhiza*, the type species and a typical *Lemna* such as *Lemna minor*. This is true of the number of roots and veins, the size of the fronds under optimum growth conditions, and the production of pigment. Indeed, Hegelmaier (9), in an appendix to his monograph on the *Lemnaceae*, suggests that the question of throwing both genera into synonymy might well be reopened, and that, if this were done, there would remain three subgenera typified by the three species mentioned above. *S. oligorrhiza* was first described from India, and later found in other parts of southeast Asia; for a long time it was not considered to be native to this country. It has since been found in California, where it was supposed to have been introduced, and in Missouri. Our culture was isolated from a tank in the greenhouse of the Biological Laboratories; its original source is unknown.

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[2] Subsequent taxonomic investigations (*cf.* below) indicate that the plant was probably a *Spirodela* species, and, if so, must have been identical with the one used in the present study.

METHODS

Culture

The plants were freed from bacterial and algal contaminants by brief immersion in an aqueous alcoholic solution of $HgCl_2$, using a concentration and time sufficient to kill some of the plants, and from then on were carried in sterile culture by ordinary bacteriological techniques. They were grown on a modified Hoagland's solution (hereafter called Medium I)[3] in Pyrex Erlenmeyer flasks or Roux bottles. Glass-

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[3] Medium I (modified Hoagland's solution):

Salt	Conc. (g/l.)	Conc. (M)
$Ca(NO_3)_2 \cdot 4H_2O$	1.180	5×10^{-3}
$MgSO_4 \cdot 7H_2O$	0.493	2×10^{-3}
KNO_3	0.506	5×10^{-3}
KH_2PO_4	0.136	1×10^{-3}
Ferric tartrate	0.010	4.9×10^{-5}
H_3BO_3	0.00286	4.6×10^{-5}
$MnCl_2 \cdot 4H_2O$	0.00362	1.8×10^{-5}
$ZnSO_4 \cdot 7H_2O$	0.00022	7.6×10^{-7}
MoO_3	0.00014	1×10^{-6}
$CuSO_4 \cdot 5H_2O$	0.00008	3.3×10^{-7}

All salts used, except the ferric tartrate, were of Reagent grade. This medium is the same as that used by Gorham (8), except that the concentrations of iron, manganese, and molybdenum are twice those in standard Hoagland's solution, because of the known high requirement of *Spirodela* for these elements. The copper concentration in later work was cut to 3.3×10^{-8} *M*, or 0.000008 g/l. (*cf.* p. 46).

distilled water was used throughout. The medium was made up and bottled, any organic substrates desired added, and the vessels then plugged with cotton and sterilized by autoclaving. Compounds not stable to autoclaving were sterilized separately by Seitz filtration. Stock cultures were kept on Medium I under low light intensity, transferred every few weeks, and also transferred occasionally to a sugar medium to detect possible contamination. (Contaminated cultures occurred rarely in the course of the investigation and were always discarded.)

Experimental flasks were inoculated with from 3 to 10 fronds and run in triplicate. Unless otherwise indicated, they were grown for these experiments under constant illumination by a combination of incandescent and mercury arc lamps at an intensity of 0.5 g.-cal./cm.^{2}/min. (*ca.* 800 ft.-candles). The mercury vapor lamps used were less effective in pigment production but more effective in promoting growth than the incandescent lamps, although the two kinds are of comparable intensity: flasks were moved about regularly to compensate for the inequalities of the light field.[4]

The air temperature in the room was maintained at 12° ± 2°C., but the high illumination raised the temperature of the solutions to 19–21°C. The effect of temperature was not included in the present study but occasional observations (*cf.* also Overton and others mentioned above) indicate a decrease of pigment formation at higher temperatures.

After 14 days, the plants were analyzed for anthocyanin content. The choice of 14 days was first made from simple observation of the depth of pigment in the plants, which appeared to have reached a constant value at this time. A later detailed study of the rate of pigment formation confirmed this impression (*cf.* Fig. 7 and Table VIII).

Pigment Extraction

In early experiments the pigment was extracted with distilled water, as follows: the plants were blotted dry, weighed, and then ground in a Waring Blendor for 4–5 min. in a measured quantity of distilled water. $ZnSO_4$ for the precipitation of proteins was added to the resulting brei to a concentration of 2%, and the whole centrifuged until clear. The absorption of the supernatant, a lavender solution of about pH 5, was then determined with a Klett-Summerson photoelectric colorimeter.

This method was subject to the following disavantages; its sensitivity was limited by the smallest amount of solution which could be handled in the Waring vessel, about 20 cc.; there was a considerable loss of solution through leakage and wetting of the vessel walls; the number of assays which could be run at once was severely limited by the equipment used; solutions were sometimes cloudy because of incomplete precipitation of starch or protein; and finally, the pigment at so low a hydrogen ion concentration was subject to occasional but uncontrollable destruction, as evidenced by color change during extraction.

Because of the greater stability of anthocyanins in more acid solution, extraction with 0.01 *N* HCl was substituted. This proved very satisfactory; indeed, it was noticed that extraction began almost as soon as the acid was added to the plant material, and this provided a clue for a further simplification. It was found that merely steeping the plants in 0.1 *N* HCl for a period of 3–4 hr. gave virtually complete extraction, obviating the necessity for grinding and centrifuging. The rate of extraction is shown in Fig. 1; the pigment extracted during 18 hr. is only about 7.5% more than that extracted during the first 3 hr., a difference well within the error of the method as a whole. Extraction is about 10% more complete with acid than with distilled water and grinding; the volume of solvent is limited only by the amount needed in the colorimeter cell (about 5 cc.); and further sensitivity is added by the fact that a solution containing a given concentration of pigment gives a higher reading in its red, acid color with the Klett No. 54 filter used than it does with its absorption spectrum at pH 5.

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[4] Light rooms have recently been completed in which it should be possible to reduce the error from this source considerably.

The standard procedure adopted was, therefore, as follows: the plants were harvested and blotted dry (the tissue is quite waxy and a reliable fresh weight is easily obtained); they were then placed in a small beaker with an appropriate amount of 0.1 *N* HCl, allowed to extract beneath a squat bell-jar for three or more hours, with occasional stirring, filtered off through paper, and the filtrate passed directly into the colorimeter cell. The amount of solvent used varied with the depth of pigmentation and the amount of plant material. In general, the proportion used was 10 cc./g. or less of plant, but if the amount of plant tissue was very small (below 0.1 g.) or the pigment very light, as little as 6 cc. of solvent gave enough solution for a reliable reading. With extremely dark plants, more solvent was used, and they were occasionally steeped for a longer period; incomplete extraction is immediately obvious.

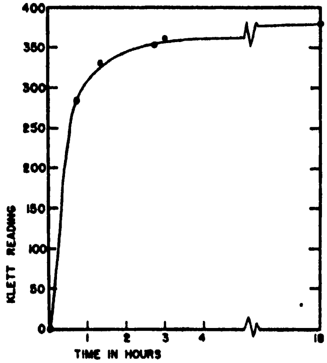


FIG. 1. The rate of extraction of *Spirodela oligorrhiza* anthocyanin in 0.1 *N* HCl. Ordinate: pigment concentration expressed as Klett readings with No. 54 filter.

Determination

The Klett No. 54 green filter has been used for all analyses. It may be seen from a comparison of the absorption spectrum of this filter with that of the pigment being studied, that the maximum transmission for the filter at 525 mμ does not exactly coincide with the nearby absorption peak for the pigment at 550 mμ (Fig. 2). It was feared that associated materials in the crude plant extracts might have yellow pigmentation which could interfere with the readings at the shorter wave length. A series of duplicate readings were therefore made using the Klett No. 54 filter and a Baird Associates interference filter, No. 7-1978A, having a narrow transmission band with its peak at 5508 Å. The results of this comparison show that the agreement between the two filters is quite constant and hence it may be assumed that the Klett No. 54 filter is entirely adequate.

By the use of dilutions of a pigment solution which gave Klett readings from 20 to 500, the absorption was found to follow Beer's law strictly. This simplifies calculations of pigment concentration. Results have been expressed throughout this paper in pigment units/10 cc. of solvent, calculated according to the following formula:

$$\text{No. of pigment units} = \text{Klett reading} \times \frac{\text{cc. HCl used}}{10}.$$

This value was then divided either by the weight of the plants (in g.) to give the concentration in "pigment units/g.," or by the number of cultures assayed, to give the amount in "pigment units/culture."

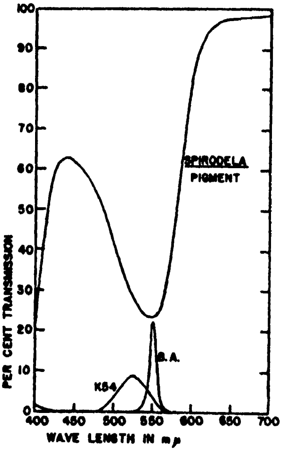


FIG. 2. Absorption spectrum of *S. oligorrhiza* anthocyanin in 0.1 *N* HCl compared with those of Klett No. 54 Filter (K54) and Baird Associates Interference Filter No. 7-1978A (B. A.). (The solution used gave a reading of 318 with K54 and 365 with B. A.) Ordinate: per cent transmission.

As yet, sufficient pigment has not been accumulated to allow of calibration of these units in actual weight of purified anthocyanin; however, the units can be recalculated to pure pigment at any future date. An approximate concentration value can be obtained from the datum that a solution containing 0.21 mg. of pure cyanin chloride (see below) in 10 cc. gives an absorption of 310 with the Klett No. 54 filter. Hence, one pigment unit is equivalent to 6.7×10^{-4} mg. of cyanin (in 10 cc. solution). While this is correct for cyanin, it is only approximate for the anthocyanin of *Spirodela*, since the absorption spectra are not identical (*cf.* Fig. 3A). Secondly, the extract of 1 g. of plants after 2 weeks' growth on Medium I also gives a reading of about 300, so that the anthocyanin content of the plants may be calculated to be 0.02–0.03% of the fresh weight. Since the dry weight is 11.9% of the fresh weight, the anthocyanin content is thus 0.2–0.4% of the dry weight.

An error of 10% has been assumed. The analytical method was, of course, much more accurate than this, but, under the experimental conditions prevailing, duplicate control flasks not infrequently showed differences of as much as 10% from one another.

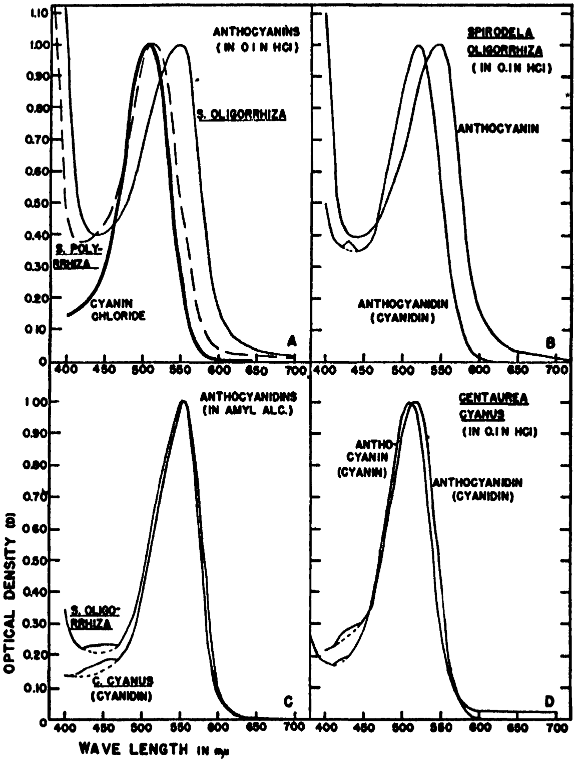
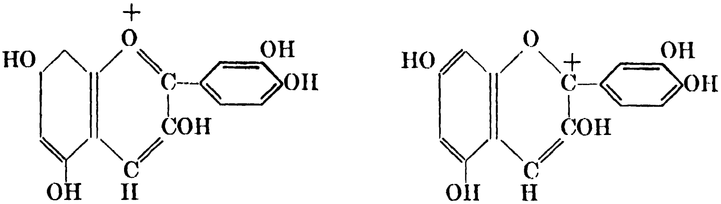


FIG. 3. Absorption spectra of anthocyanins and anthocyanidins. Ordinate: optical density (log I_0/I). All peak heights adjusted to optical density 1.0. Note the slight irregularity in the absorption spectra of the plant extracts at around 430–450 mμ, presumably due to interference by other compounds. 3A. Extracts of 2 *Spirodela* species compared with pure cyanin. The high absorptions in the near ultraviolet are probably due to accompanying flavone pigments. 3B. The change in absorption spectrum of *Spirodela oligorrhiza* pigment on hydrolysis. 3C. Absorption spectra of the aglycones of *Spirodela* and *Centaurea* pigments in amyl alcohol. 3D. The change in absorption spectrum of *Centaurea* pigment on hydrolysis (*cf.* 3B above).

Such variation was probably due in most part to fluctuations in temperature and unequal distribution of light from the two light sources used (*cf.* p. 47).

Nature of the Pigment of Spirodela oligorrhiza

The anthocyanin and its anthocyanidin (aglycone) were identified by the qualitative tests worked out by Robinson and Robinson (18,19). The anthocyanidin appeared from these tests to be identical with cyanidin, which was, therefore, prepared from flowers of *Centaurea cyanus* L. for comparison.



Oxonium formula

Carbonium formula

Cyanidin

This identity was confirmed by the absorption spectra of the two solutions (Fig. 3C), measured in a Beckman Model D photoelectric spectrophotometer.

The anthocyanin itself proved to be a diglycoside of cyanidin, as evidenced by the low partition coefficient between amyl alcohol and 1% aqueous HCl, which is not altered upon the addition of salt (19); but comparison of its absorption spectrum with that of an authentic specimen of cyanin chloride indicates either that it was a different compound, or that the solution contained extracted co-pigments which very materially altered its absorption (Fig. 3A). The absorption spectrum of cyanin prepared from *Centaurea cyanus* by the same methods used for *Spirodela* was identical, at wavelengths beyond 450 mμ, with that of the pure cyanin. The absorption spectrum of cyanin in water has also very recently been published by Commoner (3) and agrees very well with the above. On hydrolysis to the aglycone the absorption spectrum of the *Centaurea* pigment changed very little (Fig. 3D), while that of the pigment of *S. oligorrhiza* was shifted 40 mμ by hydrolysis to its aglycone (Fig. 3B). Cyanin is the 3,5-diglucoside of cyanidin; the nature and points of attachment of the two sugar residues in the *S. oligorrhiza* pigment have not as yet been determined. It is of interest to note here that the anthocyanin of the related species *S. polyrrhiza* appeared, from its absorption spectrum, to be identical with cyanin (Fig. 3A). The anthocyanin extracted from *Rosa chinensis*, which is also cyanin, was found to have its peak at 510 mμ and to agree in all respects with the curves for cyanin.

Cyanin derivatives are the most common anthocyanins in nature, and this is particularly true for leaf pigments. In an extensive survey conducted by Robinson, Price, Lawrence and others, almost 90% of the total number of genera examined had cyanidin leaf pigments, while 69% of the fruits and 50% of the flowers also contained cyanidin derivatives (14). It is at least highly suggestive that 100% of the young, pigmented leaves of monocotyledonous plants which were studied contained derivatives of cyanidin (17); this includes two species of *Tradescantia*, members of a family which is closely related to the *Lemnaceae*. However, the high proportion of such derivatives in all leaves, and the relatively small number of monocotyledons examined make it unwise to attach much importance to this fact as yet.

FACTORS LEADING TO THE PRODUCTION OF ANTHOCYANINS

Light

Light appears to be necessary for the formation of pigment, and, at least within the range used, the amount of anthocyanin formed varies directly with the intensity of the light supplied (Table I). This is true whether or not sugar is added to the medium. Continuous illumination has been employed throughout our experiments.

However, small amounts of anthocyanin can be formed in the dark. For example, in one experiment in Medium I + sucrose, when the plants had been 10 days in the light, each culture contained 24 pigment units; when the 10 days in light was interrupted after 7 days by a week in darkness the value was 39 pigment units per culture. Similarly, after 14 days in the light the culture contained 104 pigment units, while during a following period of 7 days in the dark the value was raised to 142 pigment units per culture. The dark process is relatively ineffective, however, since, in continuous light, the pigment content was, at 17 days, 218 and at 21 days, 379. Thus, although slight, the process of pigment formation in presence of sucrose in the dark is significant; this phenomenon is being further studied.

TABLE I

The Effect of Intensity of Illumination upon Pigment Formation

The plants at the lower light intensity were grown in an illuminated incubator, and those in darkness in the same incubator covered with aluminum foil. All plants assayed at the same age (17 days).

Medium	Illumination	Temp.	Growth	Pigment
	ft. candles	°*C.*	*g./cult.*	*units/g.*
I	*ca.* 800	18–19	0.66	157
	ca. 150	20	0.38	90
	none	20	0.013	(0)[a]
I+sucrose, 0.05 *M*	*ca.* 800	18–19	0.21	322
	ca. 150	20	0.39	214
	none	20	0.07	48[a]

[a] Microscopic appearance similar to inoculum.

In view of the observations of Katić and others mentioned in the Introduction it was thought of interest to examine other leaf anthocyanins in regard to the dependence of pigment formation on light. For this purpose the copper beech (*Fagus silvatica*) was selected.

Young green leaves were taken from a shaded portion of a large tree; some pigment was visible in the veins but none in the rest of the leaf. Discs having a diameter of 17 mm. were cut with a cork borer and floated on 25 cc. of solution in 50 cc. beakers, three discs to each beaker. These were placed under the lights and in the dark at the same temperature for a period of 7 days. Initial and final assays were made by steeping in 0.1 N HCl as with *Spirodela* fronds, but extraction was not complete until after 48 hr. The pigment of the copper beech is also a cyanidin derivative, in this case idaein, which is 3-galactosidylcyanidin chloride (19). Results are shown in Table II. It is clear that pigment is formed only in the light, and to a much greater extent when sucrose is added. In the dark, the amount of pigment in this material actually decreases [*cf.* Abbott (1)].

TABLE II

Effect of Light and Sugar upon Anthocyanin Formation in Discs of Fagus silvatica L.

Solution	Pigment units, total		
	Initial	7 days	
		Dark	Light
Water	55	20	75
Sucrose, 0.025 M	55	20	205

Hydrogen Ion Concentration

The modified Hoagland's solution used here has a pH after autoclaving of 4.85 (4.80 when 0.05 M sucrose has been added). A series of flasks of pH 4 to 8 was made up by adding 0.1 N HCl and 0.1 N KOH in appropriate amounts. Unpublished observations in these laboratories by Kaufman have shown that the growth of *Spirodela* was increased considerably by an increase in hydrogen ion concentration, reaching a maximum at about pH 4. However, at two weeks of age this effect was still only slight in our experiments. There was also some decrease in growth at the end of two weeks at pH 8. Data on growth and pigment concentration are given in Fig. 4. The sharp increase in pigment/g. at pH above neutrality confirms the observations of Hicks (10) on *Lemnaceae* in nature. It is not clear whether this is a direct effect of hydrogen ion concentration or the result of some associated changes in the ion balance in the medium. Since the decrease in growth at high pH in the inorganic medium was not echoed in the sugar medium, while the increase in pigment was, growth differences (see below) can hardly be considered to be the explanation.

Micronutrients

The micronutrient solution usually added to the medium was made up as 5 separate solutions and the concentrations of each (see footnote, p. 36) were varied independently.

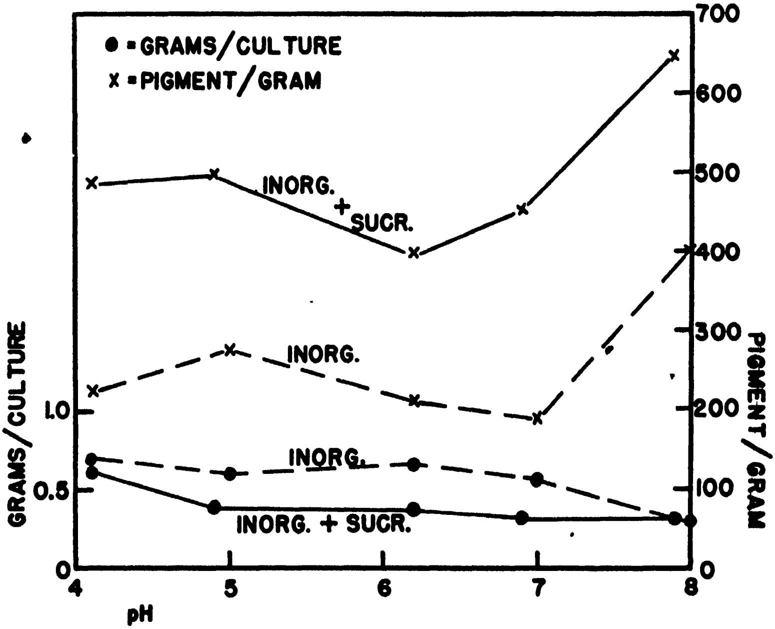


FIG. 4. The effect of pH on the growth and pigmentation of *S. oligorrhiza*, in Medium I (inorganic) with and without 0.05 *M* sucrose. Ordinates: left, growth (black dots); right, pigment concentration (crosses).

A decrease of Mn to 1/10 the normal concentration, *i.e.*, to 1.8 × 10^{-6} *M* interfered seriously with the growth and metabolism of the plants; growth was only 7–8% of that of the controls, while the fronds were small and yellowing at the edges, and anthocyanin pigmentation was uneven. The effects of manganese deprivation at this level were too severe to allow the detection of specific effects on anthocyanin production. This is in accord with the work of Saeger (20) on several of the *Lemnaceae*, including *S. oligorrhiza*.

Boron, molybdenum, and zinc, when reduced to 1/10 the concentration in our medium, showed results which were strikingly similar and apparently quite non-specific. Growth was only 15–20% of normal, while pigment was increased to a mean value of about 565 units/g. in both media, with wider variation in the case of low zinc than in the others (Table III). There is good reason to believe that this increase in pigment concentration was simply a concomitant of the decreased growth (see below). Molybdenum was also fed in higher than normal concentration, to repeat if possible the production by Warington (22) of an anthocyanin-molybdenum complex within the plant. This was not obtained at the concentration used (10 times normal), nor was there any evidence of specific interference with pigment formation by the high Mo concentration.

Copper is more difficult to discuss in quantitative terms, because it is so frequent a contaminant of distilled water and is doubtless not completely removed upon the

TABLE III

Effect of Low Micronutrient Concentrations upon Growth and Pigment Production, in Presence and Absence of Sucrose

Medium	Modification	Growth	Pigment
		G./cult.	*units/g.*
Medium I	None	0.46	194
	1/10 B	0.07	562
	1/10 Mo	0.08	563
	1/10 Zn	0.06	640
Medium I+sucrose	None	0.24	452
	1/10 B	0.05	575
	1/10 Mo	0.06	555
	1/10 Zn	0.05	512

second distillation in Pyrex glass. It is also a trace contaminant of reagent chemicals. In other experiments in this laboratory, a decrease in growth due to copper deprivation could be seen only after growth had proceeded for 3–4 weeks. Variation of copper content over a 1000-fold range resulted in no consistent effect on growth or on pigment production within the 14 day period. Increase of Cu concentration to 100 times that originally added, *i.e.*, to 3×10^{-5} *M*, was required to bring about marked copper toxicity; growth was reduced to 15–25% of normal, while pigment concentration

TABLE IV

Effect of Altered Copper Concentration in the Medium upon Growth and Pigment Production

Amounts of copper added are given as multiples of the standard concentration in Medium I (3×10^{-7} *M*). All figures except last column are results of a single experiment; all cultures aged 14 days.

	Copper added					
	100 ×	10 ×	1 ×	0.1 ×	0.01 ×	None
Medium I						
Weight: g./cult.	0.05	0.20	0.29	0.32	0.31	0.23
Pigment: units/g.	566	331	265	349	323	333
Medium I+sucrose						
Weight: g./cult.	0.05	0.23	0.20	0.20	0.25	0.19
Pigment: units/g.	560	593	417	600	588	526

again reached about 560 units/g. (Table IV). This increase in pigmentation following excess copper feeding was similar to that of the nonspecific minor element deprivation referred to above, and was very probably again merely a result of the decrease in growth.

The differences between the pigment concentrations in the standard and one-tenth standard copper solutions in Table IV were not borne out in a series of 12 subsequent experiments, which were subjected to statistical analysis. The mean values for these were, in Medium I, 273 units/g. for standard copper concentration and 293 units/g. for one-tenth standard, which is well within the limits of error. For Medium I plus sucrose they were 472 and 485, respectively. However, in the medium containing one-tenth the standard copper, the variation between individual experiments was somewhat reduced. For this reason the lower copper value was adopted for the standard medium in all subsequent experiments. Since the entire omission of added copper from the media showed no effect even after 4 weeks, the ferric tartrate, which was the only constituent of the medium not of reagent grade, was subjected to spectrographic analysis. It proved to contain about 0.1% of copper, which would correspond to the addition of 1.5 × 10^{-7} *M* copper to the final solution. For a definitive study of the effects of copper deficiency, recourse was therefore had to the use of copper-combining reagents. The results of this procedure will be discussed in a later paper.

Added Sugars

The most important and best-documented conclusion from the literature outlined above is the marked promotion of anthocyanin formation by sugar feeding or sugar accumulation. It was felt desirable, therefore, to study the role of sugars. Table V shows the effect on pigment concentration of the addition to the medium of a series of sugars and sugar alcohols. It is clear that certain sugars markedly increase pigmentation, while others do not. The sugar alcohols were quite ineffective. Of the active sugars, sucrose was by far the most effective, and this has been borne out by

TABLE V

Effect of Added Sugars and Sugar Alcohols on Anthocyanin Production

Values are means of several experiments except where starred. All data expressed as per cent of the values in Medium I.

Sugar	Sucrose		Lactose		Glucose		Fructose		Galactose		Mannitol		Sorbitol	
Conc., M.	.01	.05	.01*	.05*	.01	.05*	.01	.05	.01*	.05*	.01*	.05	.01*	.05*
Pigm units/g., per cent of control	160	167	107	93	98	91	124	144	105	99	97	76	106	119
G./cult., per cent of control	121	103	144	98	192	261	139	182	162	156	139	95	152	117

the results of a large series of experiments. In contrast, the ineffectiveness of glucose was striking. On the whole, these data agree with the results of Overton and the other early workers. Pigment was formed also in the absence of sugar, but the plants, of course, were actively photosynthetic.

The formation of pigment in sucrose medium was independent of concentration above about 0.015 *M* (Fig. 5). Since the plants were actively growing and presumably accumulating as well, this need not, of course, represent the concentration within the plant. At concentrations of 0.05 *M* and above, growth may be reduced below that of non-sugar controls. A concentration of 0.025 *M* has been adopted for later work.

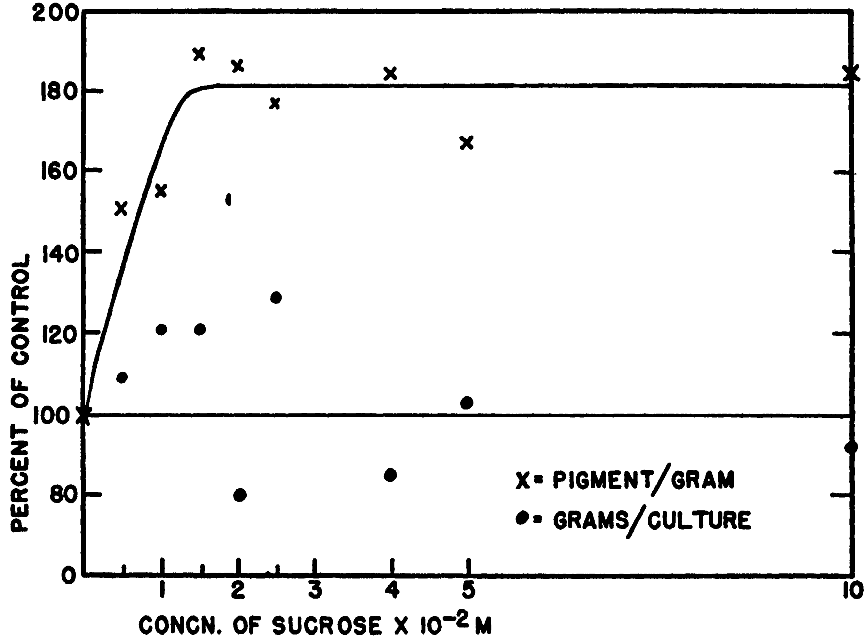


FIG. 5. Anthocyanin formation and growth as a function of sucrose concentration. No line is drawn through the growth data since, at the 14 day age used for sampling, the yield of *Spirodela* is changing so rapidly that it is subject to large variation (*cf.* Table VIII).

Rate of Growth and Rate of Pigment Formation

In two-week experiments evidence has frequently appeared that a small crop of plants is correlated with a high pigment concentration. Some reference has been made to this phenomenon above in connection with the effect of different lamps and of nutrient concentrations above or below the optimum for growth. In an experiment in which 3 Roux bottles containing the same medium were allowed to remain at different distances from the lamps without being moved for the whole 14 days, and then assayed

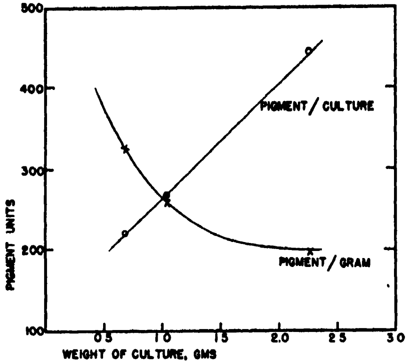


FIG. 6. Variation in amount and concentration of pigment as a function of growth. (Medium I plus glucose 0.05 M; 14 days.)

separately, the same effect was shown (Fig. 6). While the actual amount of pigment in each culture was greater as the total amount of plant material increased, the *concentration* of pigment decreased with increasing weight.

The surprising extent to which pigment production was indeed independent of growth was demonstrated in the following experiment. Flasks of 4 kinds were prepared: Medium I, the same plus 0.05 M sucrose, distilled water, and 0.05 M sucrose alone. Since the latter two solutions contained no nutrient salts, growth to any extent was, of course, precluded in these. Duplicate cultures with large, uniform inocula were placed under illumination and assayed after 6 days. The results, in terms of amount of plant and concentration of pigment, are shown in Table VI. Clearly, the concentration of pigment was much higher in the nonnutrient flasks, in which no growth had taken place. The stimulation of pigment formation by sucrose was further superimposed on the effect of growth.

Further calculations may be made from the above data, assuming that no growth took place in the nonnutrient cultures, and that the inocula were uniform. The amount

TABLE VI

Pigment Formation on Nutrient and Nonnutrient Solutions

Soultion:	Nutrient		Nonnutrient	
	Med. I	Medium I + 0.05 M sucrose	H_2O	0.05 M sucrose
Weight: G./cult.	0.38	0.22	0.17	0.16
Pigment units/cult	122	112	106	128
Pigment units/g.	318	509	624	800

of pigment initially present in each culture was calculated from the pigment concentration in the inoculum, assuming that no growth took place in the nonnutrient cultures. Hence, the amount formed during the experimental period could be determined (Table VII). In view of the large differences in the quantity of new tissue produced, the differences in the amounts of pigment produced were remarkably slight. The fact that the tissue on Medium I had more than doubled, was reflected in only a slight increase in pigment formed over that in presence of sucrose. Even in the absence of growth, the amount of pigment formed was virtually the same as on the nutrient media.

A more detailed study of growth and pigment formation was therefore made. Plants were grown in Medium I alone and with the addition of 0.05 *M* sucrose and of 0.05 *M* glucose for a period totalling 11 weeks. Pigment assays were made on samples at intervals. The data on growth in the sugar media will be discussed in detail in a later paper; in general outline, the curves obtained for the sugars are very similar to those for Medium I. These are given as logarithmic plots against time in Fig. 7. From this it can be seen that the rate of pigment production (pigment/culture), as expressed by the slope of the curve, began to fall off sooner than the rate of growth; after about 6 weeks they again became parallel. The result of this independent behavior of the two systems is shown by the curve for pigment concentration, which increases rapidly during the first few days, decreases somewhat when the rate of pigment production falls below the rate of tissue production, and finally attains a stable value when both contributing factors are once more parallel in their behavior. This is shown also in Table VIII, where selected values for the two rates of production and for the conse-

TABLE VII

Comparison of the Amounts of Pigment Formed on Nutrient and Nonnutrient solutions

Solution.	Nutrient		Nonnutrient	
	Medium I	Medium I + 0.05 *M* sucrose	H_2O	0.05 *M* sucrose
Initial wt./culture, g. (assumed)	0.16	0.16	0.16	0.16
Final wt./culture, g.	0.38	0.22	0.17	0.16
Initial pigment/culture (calculated)	37	37	37	37
Final pigment/culture	122	112	106	128
Amount tissue formed, g.	0.22	0.06	(0.01)	0.00
Per cent increase	+138	+38	+(6)	0
Amount pigment formed/cult.	85	75	69	91
Per cent increase	+230	+203	+186	+246

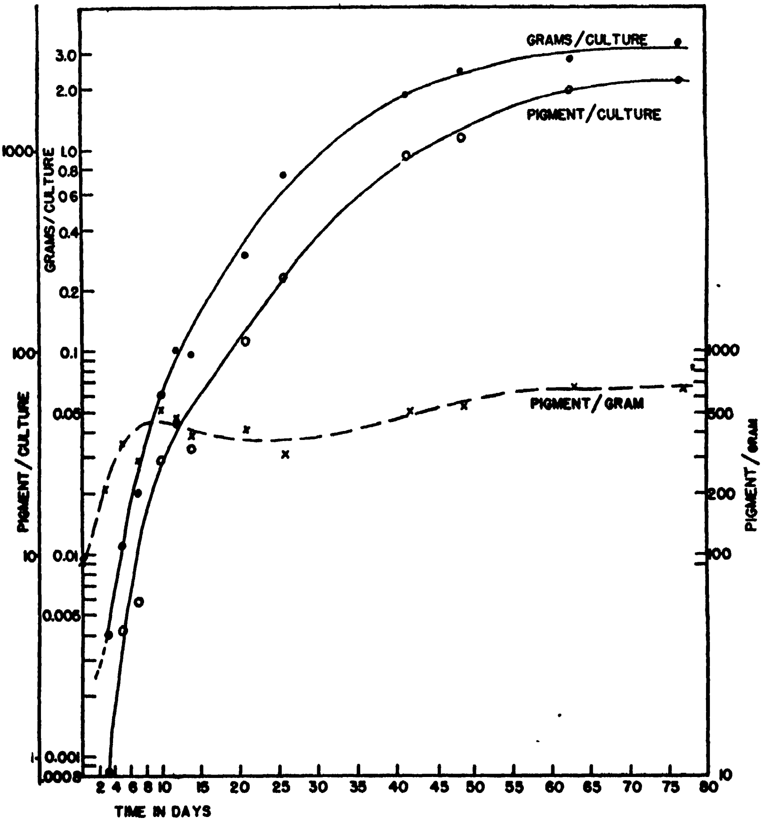


FIG. 7. Growth and development of pigment in *S. oligorrhiza* over a period of 11 weeks. See text.

TABLE VIII

Relative Rates of Change in Growth, Pigment Formation, and Pigment Concentration

Calculated from mean values derived from smoothed curves (Fig. 7).

Period	Relative rates of change (per cent change/day)		
	Pigment	Weight	Pigment conc.
days	*units/cult.*	*g./cult.*	*units/g.*
3–7	+48%	+36%	+16%
8–12	+22%	+23%	−0.28%
13–17	+12%	+16%	−2.8%
63–70	+1.4%	+0.46%	+0.44%

quent rate of change of pigment concentration are given. These calculations also bring out the relative stability of pigment concentration at the 14 day period.

DISCUSSION

The earlier investigations discussed in the Introduction show that high light intensity, low temperature, and interference with translocation by injury, all lead to anthocyanin production. These factors all favor the accumulation of photosynthetic products as well, and one possible conclusion is that sugars are necessary for the production of pigments. This view is strongly supported by the results of the "sugar-feeding" experiments of Overton and others.

The experiments reported in this paper confirm, with *Spirodela oligorrhiza* under controlled conditions, the natural and experimental phenomena previously reported. Light is necessary at some stage, and pigment production is proportional to its intensity. Small amounts of pigment can, however, be formed in the dark. The direct addition of sugars, particularly sucrose, to the medium results in increased pigment.

Interference with the growth rate may be considered to be analogous in its effects to injury in land plants. That is, to a marked extent, pigment production, which is presumably either dependent on the photosynthetic mechanism through the necessity for its products, or is itself the result of a photochemical reaction, is independent of growth. The implication of these data is that some precursor, which can be diluted by rapid growth of the tissues, is limiting, rather than an enzyme which would presumably be formed concomitantly with tissue growth. Sudden cessation of growth and the consequent accumulation of photosynthetic products may be compared with similar accumulation in a land plant in a region distal to the point of injury but on the side nearer to the area of photosynthetic production. The results in both cases are at least consistent with the idea that the accumulation of sugars is necessary for the production of pigment.

Further work on the role of sugars, and of sucrose in particular, in anthocyanin formation will be reported in a later paper. A brief preliminary account of some of this, as well as part of the above work, has already been given (4).

ACKNOWLEDGMENTS

The authors' thanks are due to Professor Sir Robert Robinson who was good enough to supply a sample of cyanin originally prepared by Willstätter, to Dr. Paul Gorham for comparison cultures of *Lemna minor* and *Spirodela polyrrhiza* from his own isolates, and to Mr. David Kaufman for spectrographic analysis of ferric tartrate. Technical assistance was contributed by Mr. Paul C. Mangelsdorf, Jr., and Mrs. Virginia Glastetter, to whom we also wish to express our thanks.

SUMMARY

1. Methods are described for the culture of *Spirodela oligorrhiza* Kurtz under controlled, sterile conditions and the quantitative assay of the plants for anthocyanin content.

2. The nature of the pigment is established as a diglycoside of cyanidin, and absorption spectra are presented.

3. Light is essential to continued pigment formation, which varies directly with the intensity. A hydrogen ion concentration above neutrality favors the production of pigment.

4. Concentrations of boron, zinc, and molybdenum below the optimum for growth, and of copper above the optimum, favor increased anthocyanin concentration by inhibiting growth.

5. The addition of sucrose to the medium stimulates pigment production. Fructose and some other sugars are active but to a lesser extent; glucose is inactive. Sucrose reaches its maximum effectiveness at a concentration in the medium of about 0.015 *M*.

6. The rate of pigment production is relatively independent of the rate of growth of the plants. This enables pigment to accumulate when the rate of growth is slowed.

7. The possibility is discussed that most of the above observations may be interpreted as due to the production of anthocyanin from sugar accumulated in the plant tissues.

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Vitamin Interrelationships in *Lactobacilli*[1]

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INTRODUCTION

The nutrilite requirements of many species of bacteria have been thoroughly investigated and growth curves have been obtained with many of the individual vitamins and amino acids in media which contained maximal amounts of all the other necessary growth factors (1). In the present experiments, the interdependence between vitamins as growth factors was studied in *L. arabinosus* and *L. casei* by setting up a series of growth curves for each vitamin in the usual type of medium, except that another required vitamin was present in various limiting amounts rather than in excess. The relationship between tryptophan and some of the vitamins was also tested in this manner. With most of the nutrilites tried, the height of the growth curve was gradually increased as the content of the limiting vitamin or amino acid was increased. In some cases, notably in tests with *p*-aminobenzoic acid (PABA) as one of the growth factors, inhibitory effects were observed. Previous studies with bacteria and yeasts have shown that antagonisms exist between certain amino acids as growth factors (2–5) and that high levels of certain single amino acids may depress growth in an otherwise adequate medium (6,7). The present study extends this concept to vitamin relationships.

EXPERIMENTAL

Methods

For experiments with *Lactobacillus casei* (A. T. C. C. No. 7469) the Landy and Dicken medium (8) was modified as previously described (9). Experiments with

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[1] A preliminary report was presented before the Division of Biological Chemistry of the American Chemical Society at Chicago, Illinois, May, 1948.

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Lactobacillus arabinosus 17-5 (A. T. C. C. No. 8014) were conducted with the nicotinic acid assay medium of Krehl, Strong and Elvehjem (10). In those studies requiring nicotinic acid in the medium, it was added at a level of 400 γ/l. In most of the experiments, Difco vitamin-free casamino acids[3] was used as the acid-hydrolyzed casein.

Stock cultures of the organisms were kept in yeast agar stabs (10) and transferred at monthly intervals. These were incubated for 36 to 48 hr. (*L. casei* at 37°C., *L. arabinosus* at 30°C.) and then stored in the refrigerator. Stabs for daily use were made from one of the stock cultures and incubated at 37°C. for 36 to 48 hr. The method of preparation of the inocula from the daily stabs has been described (9).

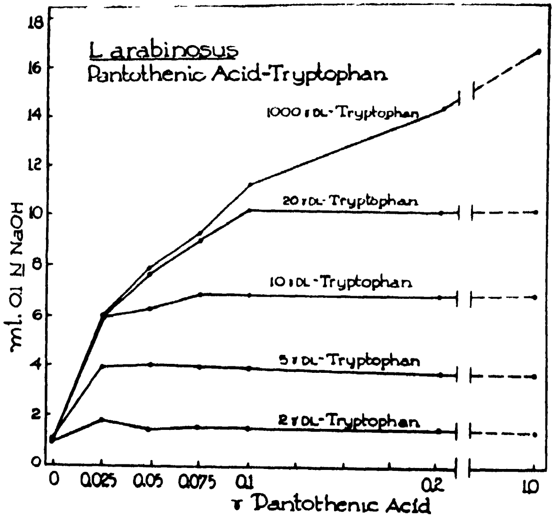

FIG. 1.

The tests were incubated at 37°C. for about 65 hr. and the acid produced was titrated with 0.1 *N* NaOH, using bromthymol blue as indicator. In a few experiments, growth was also measured turbimetrically after 24 hr. of incubation, to confirm the results obtained by titration.

The two growth factors to be studied in each experiment were omitted from the medium. Each of these was added separately to the assay tubes so that a growth curve with each substance was obtained at each level of the growth curve of the other. The volume in each tube was adjusted to 5 ml., and 5 ml. of the medium was added. With *L. arabinosus*, nicotinic acid, pantothenic acid, biotin, *p*-aminobenzoic acid (PABA), and tryptophan were tested, two at a time, in all combinations. Using *L. casei*, similar experiments were conducted with some of its essential nutrilites.

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[3] This material was generously supplied by the Difco Laboratories, Inc., through the courtesy of Dr. H. W. Schonlein.

RESULTS

Representative curves showing the types of growth relationships obtained with *L. arabinosus* are shown in Figs. 1 to 6. Since the other experiments with *L. arabinosus* and the few with *L. casei* resulted in growth curves which can be compared to the types shown in the figures, all of the combinations tested are tabulated in Table I on this basis.

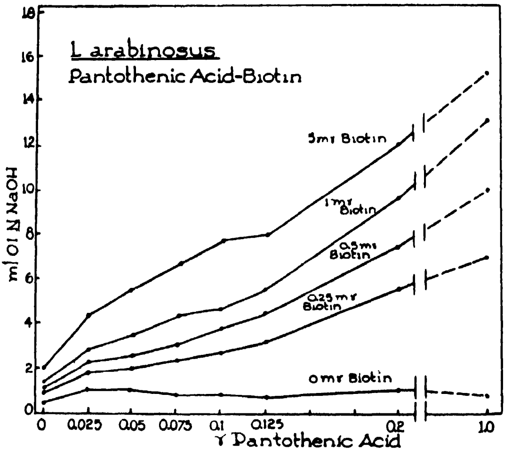

FIG. 2.

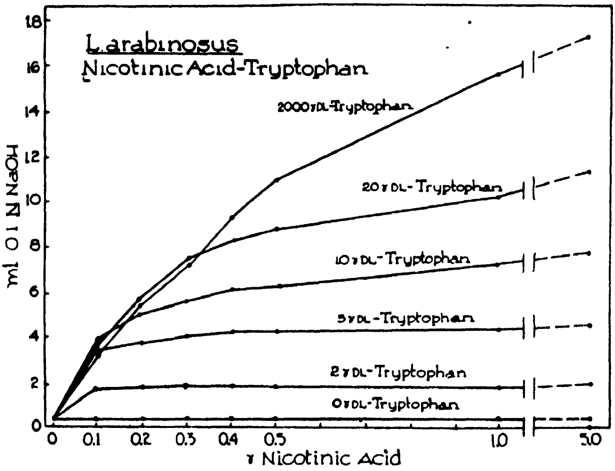

FIG. 3.

Fig. 1 shows the effect of the amount of tryptophan added to the medium on the pantothenic acid growth curves obtained with *L. arabinosus*. As more tryptophan was added the maximal growth ob-

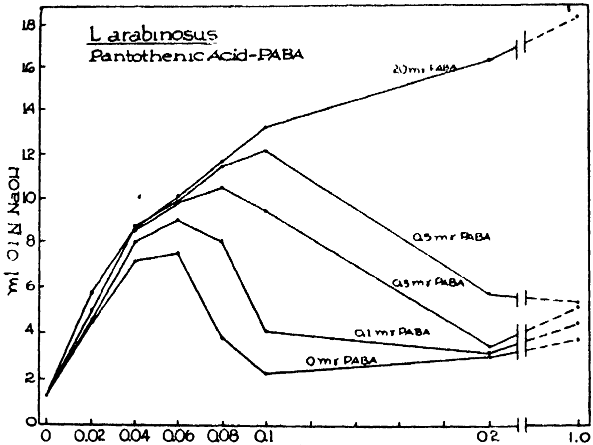

Fig. 4.

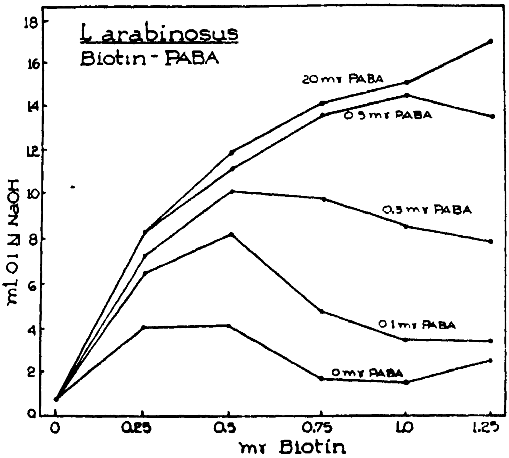

Fig. 5.

tained with excess pantothenic acid was increased. When excess tryptophan was present (1000 γ per tube), pantothenic acid became the limiting factor, and the conventional curve for pantothenic acid in a complete medium was obtained. With suboptimal amounts of tryptophan, all levels of pantothenic acid tried, either afforded the maximal growth as limited by the tryptophan present, or were fully utilized to support the same growth as was obtained with excess tryptophan. All other relationship experiments which fell into this category included tryptophan as one of the test components (Table I).

In Fig. 2, biotin and pantothenic acid utilization by *L. arabinosus* is characterized. The slope and height of the pantothenic acid curves were slowly increased as the biotin level was raised. Neither substance fully limited growth, nor allowed full utilization of the other, as did tryptophan in Fig. 1. This suggests that a close interdependence may exist between biotin and pantothenic acid, which results in slower growth with suboptimal amounts of both test substances than when an excess of one is present. Other compounds which yielded this type of result with *L. arabinosus* and *L. casei* are shown in Table I.

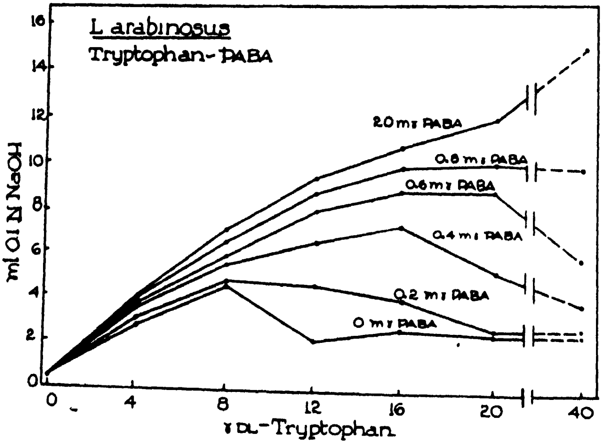

Fig. 6.

Figs. 1 to 6. Growth curves for *L. arabinosus* in the presence of limiting amounts of various growth factors.

The tryptophan-nicotinic acid curves obtained with *L. arabinosus* (Fig. 3) are somewhat similar to those shown in Fig. 1, except that excess tryptophan supported less growth with small amounts of nicotinic acid than did the limiting amounts of tryptophan. Although the degree of inhibition is small, it appears to be real, as evidenced by repeated experiments. The curve obtained with 1000 γ of tryptophan per tube was the same as the one shown with 2000 γ of tryptophan (Fig. 3). These curves were chacteristic of many of the relationships observed in tests which contained nicotinic acid as one of the test vitamins (Table I). It is possible that the compounds which gave results such as shown in Fig. 1 and Fig. 2 might have shown this same slight inhibition if the concentration of one of the substances was increased.

Figs. 4 to 6 show examples of more marked inhibition than was seen in the previous experiment. All of these contained PABA as one of the test compounds. Small amounts of PABA (even the traces in the basal medium) supported some growth of *L. arabinosus* when only a small amount of pantothenic acid, biotin or tryptophan was present (Figs. 4 to 6). Higher levels of the last three substances inhibited the growth of *L. arabinosus* with the low levels of PABA. This effect was most marked with pantothenic acid and biotin. When growth was measured turbidimetrically after 24 hr. of incubation, the curves obtained were similar to those based on acid production after 65 hr. It is interesting to note in Fig. 4 that the pantothenic acid level which supported maximum growth or acid production in each curve was increased as the

TABLE I

Types of Vitamin Relationship Curves Observed as Compared with Those Shown in Figs. 1 to 6

Organism	Fig. 1	Fig. 2	Fig. 3	Fig. 4
L. arabinosus	PA-Trypt.[a] Biotin-Trypt.	Biotin-PA NA-Biotin	NA-Trypt. NA-PA NA-PABA	PA-PABA Biotin-PABA Trypt.-PABA
L. casei	NA-Trypt. Pyr.-Trypt.	RF-PA NA-Pyr.	NA-PA NA-RF NA-Biotin NA-PGA	

[a] The growth curves for the first factor are plotted at each level of the second, as shown in Fig. 1 to 6. Reversal of the vitamins has no effect on the curves in Fig. 2, but makes the others more difficult to compare.

The abbreviations used are as follows: PA—Pantothenic acid; PABA—*p*-aminobenzoic acid; NA—nicotinic acid; RF—riboflavin; PGA—pteroylglutamic acid; Pyr.—pyridoxine; Trypt.—tryptophan.

PABA concentration was raised. The data suggest that, at suboptimal levels, the ratio of the concentration of PABA to that of the other growth factor is an important growth determinant for *L. arabinosus*. The relationship between PABA and nicotinic acid appeared to differ from this and was similar to that found in Fig. 3.

DISCUSSION

Although it is difficult to draw conclusions about metabolic processes from bacterial growth rates, the growth curves obtained with one vitamin, when another is present in various limiting amounts, may yield some information in regard to the relationship between the two compounds as growth factors.

In the presence of suboptimal amounts of two nutrilites, the growth of bacteria is limited by the compound present in the smaller concentration, relative to its requirement by the organism. Thus, 2–10 γ of DL-tryptophan limited the growth of *L. arabinosus* in the presence of more than 0.025 γ of pantothenic acid (Fig. 1), and 20 γ of tryptophan in the presence of more than 0.1 γ of pantothenic acid. It is only when tryptophan was present in excess (1000 γ per tube) that the level of pantothenic acid became the limiting factor. At any of the tryptophan levels, the smaller amounts of pantothenic acid appeared to be fully utilized, since they supported either as much growth as was obtained with excess pantothenic acid in the presence of the same amount of tryptophan, or as much as could be obtained with that level of pantothenic acid in the presence of excess tryptophan. This suggests that the metabolic relationship of the two substances is such that the low levels of pantothenic acid do not slow the growth obtained with limiting amounts of tryptophan. The utilization of other pairs of growth factors appears to involve a relationship in which the low level of one substance slows the growth obtained with limiting quantities of the second. This is exemplified by Fig. 2. At low levels, both the biotin and pantothenic acid appeared to limit growth, and each was not fully utilized in the presence of small amounts of the other.

Although the nicotinic acid growth curves with suboptimal amounts of tryptophan (Fig. 3) are similar to those of Fig. 1, the addition of excess tryptophan caused a slight inhibition of growth with the smaller amounts of nicotinic acid. High levels of each of several other growth factors had this effect on nicotinic acid utilization by *L. arabinosus* and *L. casei* (Table I).

PABA has been shown to be related metabolically to methionine, purines and pteroylglutamic acid (2,9,11,12). The growth curves in Figs. 4 to 6 show that at low levels the ratio of PABA concentration to that of other growth factors is also important. Small amounts of PABA supported some growth of *L. arabinosus* in the presence of small amounts of pantothenic acid, biotin or tryptophan. Increasing the concentration of any of the 3 latter compounds inhibited growth (Figs. 4 to 6). The antagonism between certain amino acids as growth factors (2–6) appears similar to these results involving PABA. When the data of Beerstecher and Shive (4) are plotted as tryptophan growth curves in the presence of various amounts of phenylalanine, the curves are similar to those seen in Figs. 4 to 6. These examples, as well as others, show that the ratio of the concentrations of growth factors is sometimes more important in determining growth than are the absolute amounts.

SUMMARY

Series of vitamin and tryptophan growth curves have been obtained for *L. arabinosus* and *L. casei* in media which contained limiting amounts of one other required vitamin.

Four types of relationships were found between these growth factors when both were present in suboptimal amounts.

1. Tryptophan levels usually limited growth, but allowed full utilization of small amounts of the vitamins tried.
2. Both substances limited growth and each was not well utilized in the presence of small amounts of the other.
3. Several of the curves with nicotinic acid were similar to those in Group 1, but an excess of other vitamins caused slight inhibition of growth with low levels of nicotinic acid.
4. With small amounts of PABA, low levels of pantothenic acid, biotin or tryptophan supported moderate growth of *L. arabinosus*. Increasing any of the latter three inhibited growth. The data suggest that at low concentrations, the ratio between the level of PABA and other growth factors is important for growth of *L. arabinosus*.

ACKNOWLEDGMENTS

The technical assistance of Antoinette Dingraudo, Phillis Gerhardt, Carol Haas and Janice Loeb is gratefully acknowledged.

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Isolation of L-Lyxoflavine from the Human Myocardium

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INTRODUCTION

In 1947, Sodi, Velez and Rivero Carvallo (1) isolated L-lyxose from human cardiac muscle by means of hydrolysis with cobra venom, using Drury's technique. However, it was not determined in what manner this pentose was combined with other chemical groups. The object of the present investigation is the isolation, identification, and synthesis of the compound formed by the union of L-lyxose with a flavine, which is the form in which the pentose is found in the human myocardium.

EXPERIMENTAL

1. *Isolation of 6,7-Dimethyl-1′-(L-Lyxosyl)-9-Isoalloxazine*

Ten kg. of human heart muscle were triturated with 10 l. of 95% ethyl alcohol in an electric liquefier. The resulting mass was filtered, and the filtrate, after adjusting to pH 2 with *N* HCl, was placed in a 12 liter flask connected to a reflux condenser. The solution was boiled for 2 hr. to effect acid hydrolysis. The pH was checked every 15 min. to insure maintenance at 2.0. The resulting product was filtered, and the filtrate evaporated to dryness *in vacuo* (15 mm.). The residue was taken up in 400 cc. of distilled water and filtered. After addition of 40 cc. of a saturated solution of $FeCl_3$ and neutralization to pH 7 with *N* NaOH, the mixture was filtered and the filtrate evaporated to dryness *in vacuo*. The resulting mass was extracted with 100 cc. of 95% ethyl alcohol, and the solution was filtered and then evaporated. The dry residue was extracted with 100 cc. of absolute alcohol, and the extract was filtered and evaporated to dryness. The residue was again extracted with 50 cc. of distilled water, the extract was filtered, and the filtrate chromatographed on clay. After drying, the column was eluted with a mixture of equal parts methanol, pyridine and water. The eluate was evaporated at 15 mm. Hg, and the residue crystallized, first from water and later from ethanol, to constant melting point of 278°C. Yield: 5 mg.

Anal.: Calc. for $C_{17}H_{20}N_4O_6$: C, 54.2%;H, 5.3%; N, 14.89%. Found: C, 53.9%; H, 5.1%; N, 14.2%.

Molecular weight: calculated, 376; found (Rast Method), 371.

The above extraction process was repeated 5 times to obtain sufficient material for the following procedures.

2. *Preparation of the Tetraacetate of L-Lyxoflavine*

Ten mg. of L-lyxoflavine were acetylated with 10 mg. of acetic anhydride as per Karrer (2). The product was purified in water until a constant melting point of 223°C. was obtained.

Anal.: Calc. for $C_{25}H_{28}N_4O_{10}$: N, 10.29; Found: N, 10.32.

3. *Synthesis of L-Lyxoflavine*

Eight g. of 1-amino-2-carboethoxyamino-4,5-dimethylbenzene were subjected to reductive condensation with 6 g. of L-lyxose according to Karrer (3), obtaining 4,5-dimethyl-2-carboethoxyaminophenyl-1-L-lyxosamine, with a melting point of 167°C. Yield: 1.2 g.

Anal.: Calc. for $C_{16}H_{26}N_2O_6$: C, 56.1; H, 7.6; N, 8.1. Found: C, 56.3; H, 7.9; N, 8.7.

Saponification of this material, and condensation with alloxan (0.5 g.) according to the same technique of Karrer, yielded a product which, after crystallization from water and ethanol, melted at 278°C. Yield: 260 mg.

Anal.: Calc. for $C_{17}H_{20}N_4O_6$: C, 54.2; H, 5.3; N, 14.89. Found: C, 54.6; H, 5.8; N, 15.1.

4. *Preparation of the Tetraacetyl Derivative of the Synthetic Lyxoflavine*

Ten mg. of the synthetic material were acetylated with 10 mg. of acetic anhydride as described above, yielding a product with a melting point of 223°C.

Anal.: Calc. for $C_{25}H_{28}N_4O_{10}$: N, 10.29; Found: N, 10.32.

DISCUSSION

In the present study we have succeeded in isolating L-lyxoflavine from the human heart muscle. This compound was obtained in the form of monoclinic crystals with a melting point of 278°C. Microanalysis and determination of the molecular weight established the empirical formula, $C_{17}H_{20}N_4O_6$. L-lyxoflavine is soluble in water, slightly soluble in alcohol, and insoluble in ether, acetone, benzol, and chloroform. To determine which of the 8 possible pentosidic flavines we had, the tetraacetyl derivative of the flavinic derivative (obtained from human hearts) was prepared. Its melting point (223°C.) agrees with that of the tetraacetyl derivative of lyxoflavine but does not conform with any of the other tetraacetylated derivatives. As we have previously separated

L-lyxose from the hydrolyzate of heart muscle, we assume that the product now isolated has an L-lyxo configuration. For confirmation, L-lyxoflavine was synthesized. By Karrer's method, L-lyxose was condensed with 1-amino-2-carboethoxyamino-4,5-dimethylbenzene yielding 4,5-dimethyl-2-carboethoxyaminophenyl-1-L-lyxosamine, with a melting point of 167°C. By microanalysis and determination of the molecular weight, the empirical formula $C_{16}H_{26}N_2O_6$ was established. This compound, saponified with alkali and condensed with alloxan, yielded L-lyxoflavine with a melting point of 278°C., and empirical formula $C_{17}H_{20}N_4O_6$. The tetraacetyl derivative melted at 223°C. and was shown to have the empirical formula $C_{25}H_{28}N_4O_{10}$.

All these properties are identical with those of the L-lyxoflavine prepared from human myocardium.

SUMMARY

It was found that L-lyxose, previously isolated from human heart myocardium, occurs in human myocardium in combination with a flavine, in the form of 6,7-dimethyl-1′-(L-lyxosyl)-9-isoalloxasine. This substance is analogous to vitamin B_2 except for the spatial configuration of the hydroxyl groups in the pentose chain; that is, if vitamin B_2 is D-riboflavine, our compound may be called L-lyxoflavine.

To establish the character of the isolated compound, the tetraacetylated derivative was prepared, and L-lyxoflavine was synthesized for comparison with the natural product. All tests showed that the natural product and its tetraacetate were identical with the synthetical product and its acetate.

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The Production and Some Properties of *Clostridium perfringens* Hyaluronidase [1,2]

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INTRODUCTION

In the course of studies on the production of active immunity to the gas gangrene caused by *Clostridium perfringens* it was thought desirable to ascertain the role of hyaluronidase separately from that of the α-toxin. It was, therefore, necessary to prepare the hyaluronidase free from α-toxin and in good titer.

The occurrence of a spreading factor in *Cl. perfringens* cultures was noticed in 1936 by McClean (1), and the presence of a mucinase was reported by Robertson, Ropes and Bauer (2). The production of *Cl. perfringens* hyaluronidase has been studied by Meyer, Hobby, Chaffee and Dawson (3), McClean and Hale (4), and by Rogers (5,6). McClean and Hale, and Rogers have demonstrated the adaptive nature of hyaluronidase production, but have not been concerned with the α-toxin concentration of their preparations. McClean and Hale (4) reported that their cultures of *Cl. perfringens* grown in Evans peptone plus hyaluronate produced concentrations of hyaluronidase varying from 1340 to 1620 viscosity reducing units (V.R.U.)/ml.

This paper will describe a procedure whereby culture filtrates containing 1400 V.R.U. (assay according to McClean and Hale, 1941) (4) of hyaluronidase/ml., and having only 0–10 intravenous mouse LD_{50} of α-toxin/ml., α-antitixon combining

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[1] Data for this paper are taken in part from a dissertation submitted to the Faculty of the Graduate School of the University of Cincinnati by Sanford O. Byers, June, 1944, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Cincinnati; and under a contract between the U. S. Army and the University of Cincinnati.

[2] Part of this work was presented at the May, 1944, meeting of the Society of American Bacteriologists held in New York City.

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power of only 0–0.5 units/ml. (Lb), may be obtained in a pancreatic digest medium supplemented by an easily obtainable potassium hyaluronate preparation.

The culture is maintained by pigeon passage and growth in the medium described by Logan *et al.* (7), consisting of salts, dextrin, casein hydrolyzate, tryptophan, cystine, vitamins, and fat solvent-extracted beef heart.

Hyaluronidase is produced in a medium consisting of salts, potassium hyaluronate preparation, vitamins, and pancreatic digest of beef heart. The preparation of the medium, except for the potassium hyaluronate, is described by Logan *et al.* (7).

MATERIALS AND METHODS

A. PREPARATION OF POTASSIUM HYALURONATE

1. Source Material

Human umbilical cords, stored under acetone, are ground, and the minced cords further dried by kneading in fresh acetone. The ground tissue is then spread thinly upon manila paper and exposed to air until part of the acetone has evaporated and the edges of the pieces of cord begin to darken. This partially dried cord is used as the raw material for the preparation. Ground, partially dried tissue, representing about 150 cords, weigh 1 kg.

2. Tryptic Digestion

Five hundred g. of the partially dried cord are suspended in 6.4 l. of tap water, the pH adjusted to 9.0 with 10% K_2CO_3 solution, and 40 g. of 1:100 trypsin stirred into the mixture. A liberal quantity of toluene is mixed in as preservative. Digestion is allowed to proceed at 45–50°C. for 24–48 hr., or until all the cord has been brought into solution. The fluid digest is siphoned from under a top layer of toluene, fat, and undigested blood particles, and from over a bottom layer of undigested blood particles. The digest is dialyzed through cellophane sausage casing against running tap water for 4 days, a small amount of fresh toluene being added to each casing. After dialysis the dialysis bags are suspended vertically from a horizontal rod and the toluene allowed to rise to the top. The bags are then tied off just below the upper layer of toluene, residual particles and fat, and the dialyzed tryptic digest collected by puncturing the lower part of the bag and allowing the contents to drain into a cylinder.

3. Filtration

The cloudy dialyzate is filtered to a clear, sparkling, amber colored fluid by mixing with "Supercel" (a product of Johns-Manville Co., consisting of partially vitrified diatomaceous earth) filter aid in the ratio of 20 g. of Supercel to 1 l. of dialyzate and filtering with slight suction through a thin layer of Supercel on a filter paper placed in a large Büchner funnel. Fresh paper covered with a thin layer of Supercel was used for every 2 l. of dialyzate. Filtration of the viscous dialyzate by several other methods proved to be difficult, slow and tedious. Sharples centrifugation does not remove the cloudy material, even after several passages.

4. *Precipitation of Potassium Hyaluronate*

The polysaccharide is precipitated from the cleared, dialyzed tryptic digest of umbilical cord according to the procedure of McClean (8). Ethanol (95%) is saturated with U. S. P. grade potassium acetate adjusted to pH 10 with KOH, and 1.4 vol. of the saturated alcohol added slowly, with efficient stirring, to 1 vol. of cleared dialyzate. The mixture is then allowed to stand for several hours at 4°C. After the white, stringy precipitate has settled, the fluid layer is siphoned away and the precipitate concentrated by centrifugation. The supernatant, after centrifugation, is poured off and the precipitate washed successively with ethyl alcohol, acetone, absolute alcohol, and absolute ether. It is then dried *in vacuo* over P_2O_5.

5. *Yield*

The yield from 500 g. of partially dried cord is between 10–14 g. This is equivalent to a yield of 4.5–6 g. /100 g. of oven-dried cords. The product is a white, fluffy, stringy solid. It may be powdered easily.

TABLE I

Quantitative Analysis of Potassium Hyaluronate Preparations

Analysis for	Present preparation	Theoretical values[a]	Obtained by other authors		
			McClean (8)	Meyer (22)	Kendall (21)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Ash	10.8, 10.9, 12.6	8.56	—	2.08, 4.95 (free acid)	9.2–10.2[b]
Glucosamine	32.3 (av.)	39.7	12	23.5–41.7	—
Nitrogen	2.9–3.5 (range)	3.12	4.7	3.0–3.7	3.1–3.7
Sulfur	1.1, 1.4,	0	—	Trace	—
Phosphorus	0.091	0	2	0	—

[a] Calculated on the basis of a unit formula—$KC_{14}H_{23}O_{13}N$.

[b] Converted from Na to K.

It is not obviously hygroscopic when stored in well-stoppered bottles, although it takes up water readily after it is wetted. It is only slightly sticky when pinched between the fingers. A 0.2% solution in distilled water is neutral to indicators and has a viscosity of 30–75 times that of water, at 37°C. The high viscosity indicates a long, undegraded polysaccharide molecule of high molecular weight. The viscosity is quickly reduced to that of water upon addition of hyaluronidase prepared from rabbit testicle, rattlesnake venom, or culture filtrates of *D. pneumoniae* or *Cl. perfringens*. A quantitative analysis of representative preparations of hyaluronate, comparing the present preparation with those of other authors, is presented in Table I. Ash was determined by carefully igniting the preparation, first over a free flame, and then by maintaining at red heat in a muffle furnace for several hours. The crucible was cooled, the ash wetted with a drop of concentrated H_2SO_4, and then reheated until all the H_2SO_4 was driven off. The crucible was cooled once more and a little solid $(NH_4)_2CO_3$ added to convert any $K_2S_2O_7$ into K_2SO_4.

Glucosamine was determined colorimetrically by the method of Elson and Morgan (9) against a thrice-recrystallized sample of glucosamine hydrochloride as standard. The standard was dried *in vacuo* for 20 hr. at 40°C., over P_2O_5, and gave the same color density per unit concentration as a sample of glucosamine-HCl obtained from Eastman Kodak Company. The glucosamine was liberated from the potassium hyaluronate preparations by hydrolyzing the hyaluronate in sealed tubes with 4 *N* HCl for 8 hr. at 100°C. This treatment, according to Palmer, Smyth and Meyer (10), ensures complete hydrolysis and maximal values for glucosamine.

Nitrogen was determined by the micro Kjeldahl method.

Sulfur was determined as hydrolyzable sulfur. The preparation was hydrolyzed in sealed tubes with 4 *N* HCl for 18 hr. at 140°C. The hydrolyzed material was transferred to a volumetric flask, phosphorus precipitated as $MgNH_4PO_4$ and sulfate determined according to the benzidine method as modified by Fiske (11).

Phosphorus was determined by the method of Fiske and SubbaRow (12).

B. THE PRODUCTION OF HIGH TITER HYALURONIDASE

1. *Preparation of Culture*

Stock and seed cultures are prepared as described by Logan *et al.* (7). Before use for the preparation of a seed culture, the stock culture is either passed through a 300–400 g. pigeon, or subcultured twice on two succeeding days. The seed culture is made as follows: 1 ml. of the pigeon-passed or subcultured stock is inoculated into casein hydrolyzate-beef heart broth containing 0.6% dextrin and the seed culture allowed to grow for 6 hr. at 38°C. Our laboratory strain of BP6K was found to produce as great a concentration of hyaluronidase as the best of the other 5 strains studied, and was therefore used for culture.

2. *Salts-Vitamins Supplement*

This supplement is made up as concentrated solution, and added to the medium in such quantity that the final concentration of added salts and vitamins per liter of completed medium is as follows:

$Na_2HPO_4 \cdot 12H_2O$	5.76	g.
KH_2PO_4	0.48	
$MgSO_4 \cdot 7H_2O$	0.04	
Calcium-*d*-pantothenate	0.001	
Nicotinic acid	0.001	
Thiamine	0.001	
Pyridoxine	0.001	
Pimelic acid	0.001	
Riboflavin	0.0001	

3. *Preparation of Medium*

Pancreatic digest of beef heart or veal, salts-vitamin supplement, and distilled water are added in such proportions as to give a final concentration of 4–4.5% of total digest solids. The mixture is heated to effect complete solution and the pH adjusted to 7.8 with NaOH solution. As the sole added source of carbohydrate, potassium hyaluronate preparation is dissolved to the limit of its solubility (final concentration 0.5–0.6%) in the otherwise complete medium by shaking at 34°C. The medium is then sterilized by filtration through 8 lb. Mandler candles, or by steaming for 20 min. at 1 lb. steam pressure, or by heating in boiling water for 0.5 hr. Autoclaving the medium at higher steam pressures than 1 lb. sometimes results in the appearance of an amorphous precipitate (probably polysaccharide), and media containing this precipitate yield less hyaluronidase.

4. *Inoculum*

The seed culture is centrifuged, the cell-free supernatant poured away, and the bacteria washed twice with the complete potassium hyaluronate medium to remove undesirable carbohydrate and residual traces of hyaluronidase. After the last washing, the bacteria are taken up in a volume of potassium hyaluronate medium equal to twice the original volume of seed culture. One-half ml. of this suspension is inoculated into the bottom of 40 ml. of freshly heated and cooled potassium hyaluronate medium contained in a 200 × 25 mm. tube.

5. *Incubation*

For high hyaluronidase and low α-antigen titer, the cultures are incubated at 37°C. for 120 hr.

6. *Hyaluronidase Harvesting*

The cultures are cleared by centrifugation, and may then be filtered through 8 lb. Mandler candles, or a Seitz filter with type EK serum No. 1 pads (Republic Filter Co.) There is very little loss on filtration.

7. *Storage*

Cl. perfringens hyaluronidase, prepared as described, is quite stable to storage. Fluid samples, stored at the pH of the culture filtrate (6.5–7.2) at 40°C., show no loss of activity after 3–6 months of storage. No loss occurs in the sterile filtrate on standing at room temperature for 7 days; even pure cultures stored at 4°C. for 7 days show no loss of activity. An $(NH_4)_2SO_4$ precipitate of culture filtrate showed no loss after 6 months storage at 4°C., while hyaluronidase dialyzed against glycerol in the cold has been stable with no significant loss of activity for 4 years.

C. HYALURONIDASE ASSAY

1. *Viscosimetric Test*

A viscosity reducing unit (V.R.U.) (8) is defined as that concentration of hyaluronidase which, in 20 min., will cause a loss of viscosity of substrate equal to ½ the viscosity contributed by the hyaluronic acid. The substrate used in obtaining most of the values reported here was a dialyzed and filtered (through Supercel and paper) tryptic digest of umbilical card, whose viscosity remained substantially unchanged during storage at 4°C. for 11 months. Other substrates used were solutions of potassium hyaluronate preparation. It was found most convenient to use a hyaluronate solution of concentration sufficient so that the viscosity of the buffer-substrate mixture was about 4 times as great as the viscosity of the buffer alone. Such a viscosity is effected by 0.1–0.15% solution of potassium hyaluronate preparation. The viscosity of the buffer-hyaluronate mixture is considerably less than the viscosity of an equal concentration of potassium hyaluronate in distilled water.

The buffer is made by mixing 2 vol. of *M* NaOH with 1 vol. of *M* citric acid; NaCl is then dissolved in the buffer until the solution is 1.2 *M* with respect to NaCl. Five ml. of substrate are mixed with 1 ml. of buffer, or buffer-enzyme dilution. The final pH of the 5:1 substrate-buffer mixture is 5.1.

The approximate enzyme concentration of a sample is determined in preliminary analyses. The sample for final analysis is then diluted to contain between 0.73 and 1.5 V.R.U./ml. Within this concentration range, different dilutions of the same sample do not vary more than ± 5% from the average value.

To perform the test, 5 ml. of substrate are placed in a small test tube and maintained at 37°C. in a water bath. The sample of enzyme is appropriately diluted with buffer, and the diluted enzyme is maintained at bath temperature. One ml. of diluted enzyme is pipetted into the 5 ml. of substrate and mixed by inversion. A clock started at the moment the enzyme is added records the reaction time. The 6 ml. of enzyme-substrate mixture are poured from the small test tube into an Ostwald viscosimeter, also maintained at 37°C., and viscosity measurements are repeated thereafter until the half viscosity time is reached.

Viscosimeters were permanently identified by numbers etched into the glass. Each instrument was calibrated with distilled water at 37°C.; instruments having identical flow times (59 sec.) were chosen for the work. A sufficient volume of fluid (6 ml.) was used to allow for elimination of bubbles by driving the bubble into a bulb of the instrument. Fluid was always transferred from the lower to upper bulb with the use of pressure, never vacuum.

Each time fresh buffer was prepared, the flow times of buffer plus water and of buffer plus substrate were compared with similar times for the old buffer. One of more enzymes preparations were then assayed with both old and new buffers. The new buffer was required to give the same assay as the old, within limits of the test.

The substrates used were compared similarly to the procedure used for buffers.

6. *Mucoprotein Clot Prevention Test*

When a mixture of hyaluronic acid and serum protein in solution is acidified with acetic acid, a precipitate consisting of a fibrous "mucin clot" is formed; if the hyaluronic acid has been previously hydrolyzed by hyaluronidase, the clot is absent. This reaction has been quantitated, as an assay for hyaluronidase by McClean (8). Our routine assay for hyaluronidase activity was always the viscosity reduction test. The M.C.P. test was used only to characterize the activity of the two standard enzyme preparations used in the estimation of antihyaluronidase, and in testing formalized antigens.

A mucoprotein clot prevention unit (M.C.P. unit) of hyaluronicase is sufficient enzyme so that, after incubation with the hyaluronic acid-serum protein substrate for 20 min. at 37°C., the subsequent formation of a clot is prevented. Appropriate dilutions of the sample to be tested are made in a final volume of 0.5 ml. of distilled water in small serological test tubes. One ml. of substrate is added to each tube, and to a distilled water control, and the viscous substrate mixed by inversion. At the end of the incubation period the tubes are placed in ice water for 5 min. to stop enzyme action. After cooling, 0.5 ml. of 1 N acetic acid is added and the tubes are shaken. A clot of stringy precipitate will appear in those tubes contairing less than 1 M.C.P. unit of hyaluronidase. The greatest dilution of enzyme still preventing the appearance of a clot is taken as the end point; the original sample is said to contain a concentration of M.C.P. units of hyaluronidase equal to the number of times it was diluted in the end point tube. The substrate is a mixture of potassium hyaluronate preparation and normal horse serum, in the proportions of 1 vol. of 0.2% water solution of potassium hyaluronate, 1 vol. of a 1:10 dilution of normal horse serum in 0.9% saline, and 2 vol. of distilled water. Since many heterologous sera inhibit hyaluronidase action, a number of horse sera were tested until a serum was found which (by viscosity test) did not inhibit *Cl. perfringens* hyaluronidase in any degree. This serum, sterilized by filtration and stored at 4°C., was used as a source of serum protein.

McClean (8) reports approximate equivalence of 1 V.R.U. to 8.3, 13.2, and 15.1 M.C.P. units of *Cl. perfringens* enzyme, respectively. We have obtained ratios of 1 V.R.U. to 14, 7.9, and 8.0 M.C.P. units, respectively.

D. ANTIHYALURONIDASE ASSAY

1. *Principle*

If a sample of hyaluronidase be incubated with a sufficient quantity of antihyaluronidase, its power to prevent the appearance of a "mucin clot" in the mucoprotein clot prevention test will be lost. The appearance of a clot, therefore, serves as an indicator of an effective antihyaluronidase, and may be quantitated as a measure of antihyaluronidase activity.

2. Preparation of Standard Enzyme

Two standard hyaluronidase preparations were used in the course of the work. In earlier tests, the standard consisted of an $(NH_4)_2SO_4$ precipitate, prepared as follows: 1620 ml. of mixed culture filtrates from strains BP6K and BG6, averaging approximately 110 V.R.U./ml., was precipitated by saturating with $(NH_4)_2SO_4$. The precipitate was skimmed off, dried *in vacuo* over P_2O_5, and stored *in vacuo* over P_2O_5 at 4°C. The yield was 1.14 g. of material assaying 50 M.C.P. units/mg. In later tests, a *Cl. perfringens* strain BP6K filtrate, which had been concentrated by dialysis against glycerol, was used. It contained 2400 M.C.P. units/ml.

3. Technique of Assay

A fixed concentration (4 M.C.P. units) of standard enzyme was tested against 2-fold dilutions of unknown serum in physiological saline. Each of the serial dilutions of serum were incubated for 0.5 hr. in a 37°C. water bath with an equal volume of standard enzyme dissolved or diluted in distilled water. One volume of the incubated mixture of serum and standard enzyme was then allowed to act upon one volume of substrate for 20 min. at 37°C. as in the M.C.P. test for hyaluronidase activity. The antihyaluronidase titer of a serum was taken to be the highest dilution of that serum which negated the clot-preventing action of the standard quantity of hyaluronidase.

Three controls were performed concurrently with each assay of unknown serum. (1) A sample of normal horse serum used in the M.C.P. assay substrate was tested for contiuned absence of interference; (2) a mucin clot prevention assay was run on the standard enzyme to detect loss of activity; and (3) a standard antiperfringens horse serum was titrated to detect changes in combining power of the standard enzyme. Apparent fluctuations of activity or combining power from one test to another was not greater than plus or minus one tube (100%). No consistent loss of activity or combining power was demonstrated, since a low assay in one test might be followed by a normal or high assay on the next. In addition to these three controls, the viscosity of the potassium hyaluronate preparation was frequently checked. Solutions of potassium hyaluronate showing as much as 5% loss of viscosity from initial values were discarded.

All tests of antihyaluronidase titer during the course of immunization on a single animal were carried out at the same time, against the same enzyme and on the same hyaluronate substrate.

RESULTS

A. STRAIN

The strains of *Cl. perfringens* examined for hyaluronidase production were BP6K (our laboratory strain), BP364, and BP365 (from the Wellcome Physiological Research Laboratories), WX (from Dr. G. B. Reed), 1508Ad (from Dr. Ivan C. Hall), and BG6 (Lilly Research Laboratories).

Strains 1508Ad and WX were found not to produce hyaluronidase. A total of 33 subcultures of 1508Ad in 4.5% Bacto-Tryptose medium containing potassium hyaluronate were examined. No hyaluronidase activity was demonstrated in any culture of strain 1508Ad despite a moderate increase in virulence occurring with intermediate pigeon passage. Although growth takes place in media containing potassium hyaluronate as sole added source of carbohydrate, the viscosity conferred upon the medium by the presence of hyaluronate is not destroyed by the culture. Since only a small fraction of a viscosity reducing unit of hyaluronidase, when acting over a long time, is sufficient to reduce the viscosity of hyaluronate solution, it is apparent that 1508Ad, in our hands, does not secrete hyaluronidase into the medium at any stage of its growth. A smaller series of subcultures of strain WX was examined, with similar negative findings.

Strains BP364 and BP365 both produce substantial concentrations of hyaluronidase, though somewhat less than BP6K.

Strain BG6 is an active hyaluronidase producer. Its production, on suboptimal media, is equal to that of strain BP6K. These two strains were not compared on the optimal medium.

B. NITROGEN SOURCE

Strain BP6K produces more hyaluronidase when supplied with an optimal concentration of nitrogenous nutrients (as indicated by growth and α-toxin production) than when the nitrogen supply is suboptimal.

TABLE II

Effect of Beef Heart Digest Concentration on Hyaluronidase Production

Digest concentration in the medium	Hyaluronidase V.R.U./ml.
1.0%	300
3.0%	773
4.5%	1270

Potassium Hyaluronate—0.6%.
Incubation time—65 hr.
Temperature—37°C.

This is shown in Table II, where hyaluronidase concentration is seen to increase with increasing concentration of beef heart digest in the medium, up to 4.5% Higher concentrations of beef heart digest were not systematically investigated for effect on hyaluronidase production.

Such higher concentrations usually have an adverse effect on growth and α-toxin production.

As exemplified in the data in Table III, pancreatic digests of veal or beef heart provide the greatest yields of hyaluronidase, while papain digests, Bacto-Tryptose, and casein hydrolyzate are less effective, in this order.

TABLE III

The Antigen Content of Cl. perfringens filtrates

Carbohydrate: 0.6% potassium hyaluranate.
Incubation: 60+ hr. at 37°C.

Medium	Strain	Hyaluronidase	α-Toxin		α-Antitoxin combining power. Lecithinase test
			Lecithovitellin test	Mouse IP	
		V.R.U./ml.	*LD_{50}/ml.*		*Lb units/ml.*
4.5% Tryptic digest of veal	PB6K	1380	Trace	<2	<0.05
		1400	Trace	2	<0.05
4.5% Tryptic digest of beef heart	BP6K	1371	<1.0	<1	<0.05
		1389	<1.0	<1	<0.05
3.8% Papain digest of beef heart	BP6K	685	<1.0	<1	<0.05
		742	<1.0	<1	—
Casein hydrolyzate 1600 mg. N/l. medium	BP6K	160	<1.0	<1	—
		200	<1.0	<2	—
4.5% Bacto-tryptose	BP6K	630	—	<1	<0.05
		620	—	<2	
	BP365	450	0.1	<1	—
		390	0.1	<1	—
	BP364	265	Trace	<1	—
		190	Trace	<1	—

C. CARBOHYDRATE SOURCE

The highest concentrations of hyaluronidase are obtained when the potassium hyaluronate preparation is the sole added source of carbo-

TABLE IV

Effect of Potassium Hyaluronate Content of Medium on Hyaluronidase Production 60 hours Incubation at 37°C.

Nitrogen source in medium	Potassium hyaluronate	Hyaluronidase concentration
	per cent	*V.R.U./ml.*
Casein hydrolyzate	0.1	25
	0.25	61
	0.50	150
Beef heart digest	0.1	100
	0.25	432
	0.50	1128

TABLE V

Effect of Different Carbohydrates on Hyaluronidase Production

Nitrogen source, casein hydrolyzate					Hyaluronipase concentration
Carbohydrate source					
Potassium hyaluronate	Dextrin	Glucose	Glycogen	Maltose	
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*V.R.U./ml. of medium*
0.5					388
0.5	0.1				360
0.5		0.1			160
		0.5			0
0.5			0.06		282
0.5			0.25		205
0.5	0.62				175
0.5	0.25				164
0.5		0.52			136
0.5		0.25			55
		0.25			0.4
	0.6				66
				0.6	25
		0.6			7
Nitrogen source, 4% tryptic digest of beef heart					
0.6					800–1400
	0.6				125
				0.6	45
		0.6			56

hydrate. As shown in Table IV, increased concentrations of polysaccharide result in increased yields, up to 0.5% of hyaluronate in the medium. The presence of other carbohydrates, especially glucose or maltose, together with the hyaluronate sharply reduces hyaluronidase production; production is least in media from which hyaluronate has been omitted and some other carbohydrate added. Data demonstrating the decreased yield of hyaluronidase resulting from the addition to the medium of monosaccharide, disaccharide, or polysaccharide other than hyaluronate are set forth in Table V. When used as sole carbohydrate sources, washed dextrin or glycogen permit BP6K to produce 100–300 V.R.U./ml., while glucose only yields 1–10 V.R.U./ml. of culture filtrate. Inocula from highly virulent BP6K cultures almost always produced at least a minimum of 1–2 V.R.U./ml., even when limited to an unfavorable combination of nitrogen and carbohydrate sources, that is, casein hydrolyzate and glucose.

D. INCUBATION TEMPERATURE

Satisfactory yields may be obtained when cultures are incubated at 34–40°C. The results at 42°C. are somewhat poorer.

E. INCUBATION TIME

When potassium hyaluronate preparation is used as sole carbohydrate source, tryptic digest of veal or beef heart as nitrogen source, and a virulent culture of our laboratory strain of BP6K as inoculum, the concentration of hyaluronidase activity in the medium increases rapidly for the first 15 hr. and more slowly thereafter until a maximum is reached at 60–100 hr. After this time the concentration falls off slowly, about 25% loss occurring between 100 and 300 hr. The course of hyaluronidase production in media containing tryptic digest of veal and beef heart is presented graphically in Fig. 1. α-Antigen concentration reaches a maximum between 6–17 hr., and then falls off to very low levels after 60 hr. Since hyaluronidase concentration is still high at 120 hr., harvesting at this time allows ample margin for the complete, or nearly complete, disappearance of α-antigen. The antigen content of filtrates of cultures utilizing various nitrogen sources and incubated for at least 60 hr. is given in Table III.

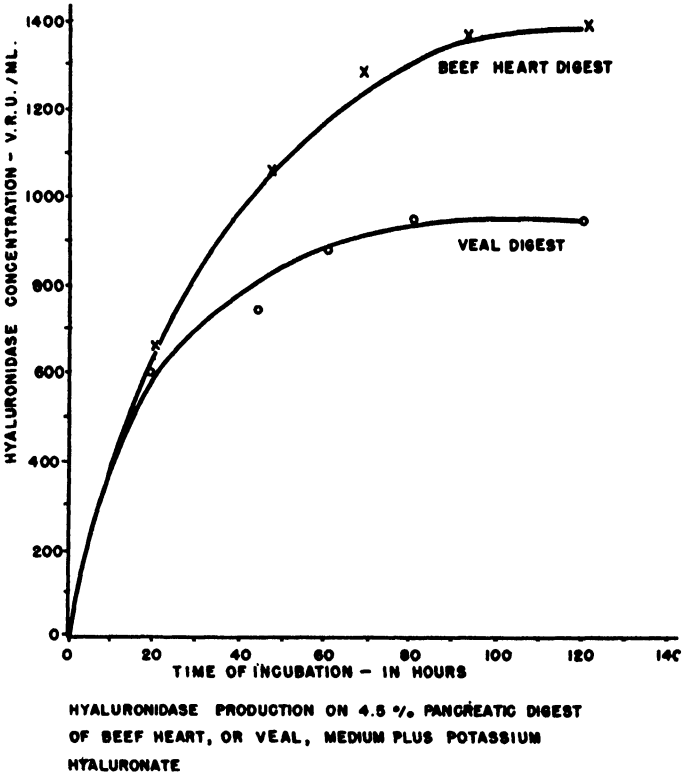

FIG. 1. Hyaluronidase production on 4.5% pancreatic digest of beef heart, or veal, medium plus hyaluronate.

F. FORMALDEHYDE INACTIVATION OF HYALURONIDASE

After reaction for 3 weeks at 34°C. with 0.2% formaldehyde, filtrates lost 95–100% of their original activity as measured by M.C.P. tests (8). Since hyaluronidase is stable to storage under the same conditions of time and temperature, it is evident that formaldehyde was able to inactivate the enzyme. The formaldehyde-inactivated hyaluronidase retains its antihyaluronidase combining power and after injection into humans and animals, gives rise to circulating antihyaluronidase.

G. EFFECT OF X-RADIATION ON HYALURONIDASE

Samples of culture filtrate were submitted to the action of soft X-rays of greatest intensity at the K_α line of copper, or 1.54 Å wavelength, and acting at a distance of 3 cm. from the focal spot of the copper target. Before and after irradiation, enzyme potency was estimated by the viscosimetric test. Unirradiated filtrates, kept at the same temperature as the irradiated samples, were used as controls. As much as 4 hr. exposure failed to inactivate filtrates from BP6K cultures grown in casein hydrolyzate medium plus ground defatted beef heart. However, when active material was purified by alcohol precipitation at its isoelectric point at −10°C. (13), a similar radiation dose caused 90% loss of activity.

H. FLOCCULATION OF ANTISERA BY HYALURONIDASE

The culture filtrates containing hyaluronidase in the virtual absence of α-antigen produce particulation and flocculation with a refined specific *Cl. perfringens* horse antiserum, Lederle No. 2147 F, preserved with glycerol. (The flocculation has been confirmed by Mr. C. H. Parsons of Lederle Laboratories, using 2 different antisera.) Varying amounts of antiserum are added to a constant amount of undiluted culture filtrate and incubated at 40°C. The tube which first shows particulation or flocculation is taken as the end point, or optimal proportions point. Table VI shows the optimal proportions points for filtrates of different hyaluronidase concentrations. It is seen that the ratio of hyaluronidase concentration to serum required is approximately constant, and, for this serum, equal to about 5.3×10^{-3} ml./428 V.R.U. of hyaluronidase. The supernatant fluid at the optimal propor-

TABLE VI

Optimal Proportions Points for Flocculation of Hyaluronidase Filtrates with Lederle Serum No. 2147F

Hyaluronidase concentration in the filtrate	Filtrate used	Dilution of serum	Diluted serum at the optimal point	Original serum/1 ml. of filtrate	Original serum/400 V.R.U.	Ratio of values in column 6 to 0.00533
V.R.U./ml.	*m.l*		*ml.*	*ml.*	*ml.*	
428	2	1:75	0.7	0.00466	0.00466 =	0.8743
428	3	1:50	0.8	0.00533	0.00533 =	1
428	3	1:10	0.175	0.00583	0.00583 =	1.094
800	3	1:5	0.16	0.01066	0.00533 =	1
1150	3	1:5	0.22	0.0154	0.00536 =	1.006

tions point is almost, or entirly, free from hyaluronidase activity, while such activity can be demonstrated in tubes containing an insufficeint quantity of serum. In a typical flocculation test 3 ml. of culture filtrate assaying 425 V.R.U./ml. were used in each tube. In this test, initial particulation occurred at 15 min. in the tube containing 0.18 ml. of serum. The next closest tube flocculated in 45 min.

I. ANTIGENICITY OF HYALURONIDASE

The antigenicity of hyaluronidase, or formaldehyde-treated hyaluronidase, was determined by injecting suitable quantities (usually 1 ml.) subcutaneously into humans or animals. Blood samples were taken before each series of injections, at intervals during the course of injections, and afterwards. The antihyaluronidase titer of a given serum was considered to be the highest saline dilution of that serum which was capable of preventing the action of a fixed amount of hyaluronidase upon its substrate. Each of the serial dilutions of serum was incubated for 0.5 hr. at 37°C. with 4 M.C.P. units (8) of enzyme, and the mixture then allowed to act upon 1 ml. of hyaluronic acid-protein substrate for 20 min. at 37°C., after which acetic acid was added to produce a mucoprotein clot. The highest dilution showing a clot, and therefore indicating an excess of antihyaluronidase, was taken to be the end point of the reaction. Each antigen assay is controlled by a standard antiperfringens horse serum titration, to detect possible loss of combining power of the enzyme preparation, and by a M.C.P. assay of the activity of the standard enzyme.

All the species tested respond to the subcutaneous injection of hyaluronidase, or formaldehyde-treated hyaluronidase, by the production of antibodies, although some individuals of each species fail to respond. Rabbits produced about 16 units; mice, 32 units; dogs, 4–30 units; and man, up to 128 units. Hyaluronidase, when used to hyperimmunize guinea pigs, gives rise to 128–256 anti-M.C.P. units/ml. of serum These guinea pigs, however, are not protected against *Cl. perfringens* type A infection; nor does hyperimmunization with hyaluronidase increase the protection of guinea pigs suboptimally immunized with normal toxoid.

A total of 119 human volunteers received courses of injections with either fluid, or alum-precipitated *Cl. perfringens* toxoid. The toxoid was rich in α-antigen, and contained about 100 V.R.U. of hyaluronidase (or toxoided hyaluronidase)/ml. The number of positive responses to a course of 3 injections at 21 day intervals was 91, or 76%. Twenty-eight failed to respond; of this group, 14 received only a single injection. Only 11, or 9.2%, showed circulating antihyaluronidase previous to injection, when tested as described. With these 11 individuals, increase of titer was taken as evidence of positive response. The human response to fluid toxoid, and to alum-precipitated toxoid, respectively, is graphed in Figs. 2 and 3.

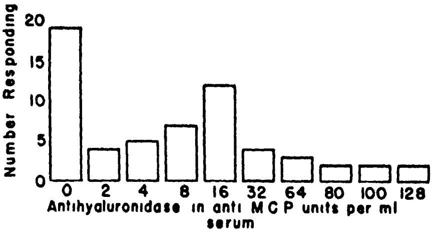

FIG. 2. Human response to fluid toxoid. The bars show the number of subjects with maximum response at the indicated levels.

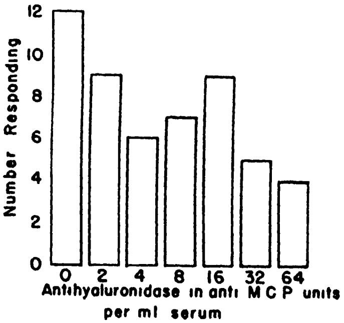

FIG. 3. Human response to alum toxoid. The bars show the number of subjects with maximum response at the indicated levels.

DISCUSSION

A. POLYSACCHARIDE PREPARATION

The method of preparation of potassium hyaluronate insures a high yield of purified polysaccharide of very good or very high viscosity. A large yield of substrate is a matter of practical importance in the production of large quantities of hyaluronidase. Hadidian and Pirie (14) have recently shown that repeated water extractions of umbilical cord fail to extract all of the hyaluronate. Although the method does not insure that the preparation is solely potassium hyaluronate, it is probable that the viscosity of solutions of the preparation is chiefly, if not solely, due to hyaluronate. That it is mainly hyaluronate is indicated by the fact that solutions of the preparation are rapidly reduced to the viscosity of the pure solvent by culture filtrates from *D. pneumoniae* grown both in a complex medium, and in the improved semi-synthetic medium of Badger (15) supplemented by potassium hyaluronate. Meyer, Hobby, Chaffee and Dawson (3,16) have shown that pneumococcus enzyme is specific for hyaluronic acid, mucoitin sulfuric acid (the monosulfuric ester of hyaluronic acid) and a trisulfuric acid ester prepared from hyaluronic acid. Their pneumococcus enzyme did not attack glycogen, starch, chitin, ovomucoid α, serum mucoid, a neutral polysaccharide from pig mucosa, chondroitin sulfuric acid, or heparin.

B. EFFECT OF VARIOUS CARBOHYDRATES UPON HYALURONIDASE PRODUCTION

Cl. perfringens produces hyaluronidase proportionally to the amount of hyaluronate in the medium up to 0.5%. This result is similar to that found by Rogers (5), and adduced by him as evidence of true adaptation to hyaluronate. This author reports also that the addition of glucose to hyaluronate medium completely suppressed hyaluronidase production by *Streptococcus* C7, an effect which he attributes to the low pH induced by the organisms in this medium. Findings in the present paper indicate that other carbohydrates added to hyaluronate medium reduce greatly the concentration of hyaluronidase which *Cl. perfringens* is otherwise capable of producing. Since polysaccharides, such as dextrin or glycogen, show less inhibitory power than monosaccharides, it seems possible that the availability of carbohydrates other than hyaluronate may be important, as well as pH effects. Most of the growth of the culture may take place at the expense of the more available carbohydrate.

C. FORMALDEHYDE INACTIVATION OF HYALURONIDASE

Previous literature mention of this subject is confined to McClean's (1) original report in 1936 of "diffusing factor" in *Cl. perfringens* toxin. At that time he concluded that formalin exerts no destructive influence on diffusing factor. Without further evidence on the subject, it is difficult to suggest an explanation of the difference between McClean's results and our own.

D. INACTIVATION BY X-RADIATION

Foreign protein may possibly protect the perfringens enzyme against inactivation by X-radiation, since purified samples are affected, while crude preparations are not. Experiments performed in this laboratory show that pneumococcus hyaluronidase from cultures grown in a semi-synthetic medium (15) supplemented with potassium hyaluronate is readily inactivated by X-radiation. The ability of foreign protein to protect against enzyme inactivation by X-ray has been clearly shown by Tytell and Kersten (17) in the case of the enzyme urease.

E. FLOCCULATION OF HYALURONIDASE BY ANTISERA

The precipitation of *Cl. perfringens* hyaluronidase by antisera is consistent with the reported precipitation of testicular hyaluronidase (18), and of pneumococcus hyaluronidase (19). This flocculation might be of value in the purification and further study of the enzyme. However, in view of the use of preparations containing antigens and enzymes other than hyaluronidase, sight should not be lost of the possibility that the precipitation may be caused by extraneous antigenic material, other than α-antigen, and that the variation of optimal proportions point with hyaluronidase concentration may be merely coincidental.

F. ANTISERUM AND NORMAL SERUM INHIBITION OF HYALURONIDASE

The work on inhibition of hyaluronidase by normal sera has recently been discussed and extended by Dorfman, Ott and Whitney (20). The necessity for sharp end points in the mucoprotein clot prevention assay, and the turbidity assay, requires the presence of more than minimal detectable concentrations of hyaluronidase. The excess hyaluronidase may mask a small inhibition. Since the amount of nonspecific inhibition varies greatly in different sera, it is difficult to determine small amounts of specific inhibition precisely. Larger amounts of specific inhibition are more easily titrated, as the nonspecific inhibition is only rarely apparent, against 4 M.C.P. units of hyaluronidase, even as low as the third serial dilution of serum, or 1:8. At higher dilutions, antisera inhibit specifically, without cross-neutralization.

In the human series, even the lowest positive response is equivalent to power to neutralize many minimal skin-spreading doses of hyaluronidase.

G. ASSAY OF HYALURONIDASE AND ANTIHYALURONIDASE

The apparent activity of a given enzyme preparation may vary with type and concentration of buffer, pH, substrate preparations, salt concentration, and perhaps other factors. Since neither enzyme, substrate, nor antisera were exchanged with other laboratories for assay, no precise comparison of the values reported here can be made with those reported by other laboratories. Even values obtained at widely separate times in the same laboratory possibly may not be exactly comparable because of change of substrate, more facile technique, variation in salt concentration in the reaction mixture, or unknown variations. Because there is no accepted hyaluronidase standard, the units reported in this paper can be compared only approximately with values previously published. The comparisons within this paper are valid for the following reasons: (1) assays connected with each factor investigated (incubation time, carbohydrate source, strain, *etc.*) were done at about the same time, with the same buffer and substrate, (2) stored samples of enzymes gave closely similar assays over a period of 4 years, and (3) substrates and buffers were compared and controlled by checking with the same enzyme each time an experiment was run.

It was necessary to dilute the enzyme preparations of high potency reported in this paper 1000 times with distilled water to obtain a sample with an optimal flow time in the V.R.U. test. It must be presumed that such an enzyme preparation is considerably more concentrated than one which may be diluted only 10 times, even though the viscosimetric substrates used in the two assays are not identical.

The concentration of salts used in the present viscosimetric reaction mixture is higher than that used by McClean (8), and may have the effect of depressing V.R.U. values as compared with those previously reported. The high concentration of salts also has the effect of minimizing variations in salt content of filtrates to be assayed. The final pH of the viscosimetric substrate-buffer mixture was 5.1. This is higher than the optimum of 4.6 reported by McClean(8) for citrate-phosphate buffer. This pH should also tend to depress the viscosimetric unit values as compared with those reported by McClean.

The uncertainties of the M.C.P. assay are at least as great as those of the viscosimetric test. When used as a means of antisera assay, the M.C.P. test is more rapid than the viscosimetric test, although less sensitive. Since it was necessary to examine many antisera in connection with a wartime program, the use of the V.R.U. test would have entailed a prohibitive expenditure of time and the M.C.P. antisera test was therefore used. The changes in titer which occur during the course of immunization of animals or humans are of such magnitude that the M.C.P. test is quite adequate to follow the course of the anti-hyaluronidase content of the serum.

SUMMARY

1. A method for the preparation in high yield of purified polymerized hyaluronate from umbilical cords is described.

2. A procedure is described whereby 1000–1500 Viscosity Reducing Units per ml. of *Cl. perfringens* (Type A) hyaluronidase may be obtained in culture filtrates substantially free from α-antigen.

3. Hyaluronidase so produced is relatively stable on storage, may be inactivated by formaldehyde, may be inactivated by soft X-radiation, when purified, and will particulate and flocculate *Cl. perfringens* (Type A) specific antisera.

4. Hyaluronidase and hyaluronidase inactivated by formaldehyde is antigenic in humans, dogs, rabbits, guinea pigs, and mice.

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The Isolation of the Coenzyme of Phosphoglucomutase

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INTRODUCTION

Previous papers (1,2) reported the existence of a coenzyme for phosphoglucomutase and its probable identity with glucose-1,6-diphosphate. The isolation and properties of the substance and its role in animal tissues are now described.

The rate of the reaction: glucose-1-phosphate → glucose-6-phosphate is, under certain conditions, proportional to the concentration of coenzyme, which can thus be easily estimated (Fig. 1).

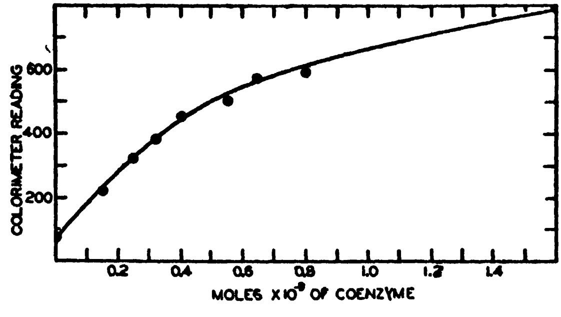

FIG. 1. Relationship between the amount of coenzyme and the activity of yeast phosphoglucomutase.

During a search for the best starting material, and following the observation of Kendal and Stickland (3) that the phosphoglucomutase of rabbit muscle is activated by fructose diphosphate, this substance was tested. The sample used was found to contain considerable amounts of coenzyme. It was next observed that, during the incubation of yeast with sugar, phosphate and ether, as described by Neuberg and Lustig (4) for the preparation of fructose diphosphate, the coenzyme content increased enormously.

The separation of these two substances proved to be very difficult. For instance, a preparation which had been purified by barium, lead and alcohol fractionation, gave the same relation of coenzyme to fructose as the starting material. The only procedure by which the fructose ester could be removed was the destruction by heating at alkaline reaction. Large amounts of inorganic phosphate are then liberated, since the amount of coenzyme is only about 0.5% of the other phosphoric esters. The removal of the inorganic phosphate with magnesia mixture often resulted in great losses by coprecipitation. This difficulty was finally overcome, and, after purification of the product by lead, barium, and acetone fractionation, a substance of about 70% purity was obtained.

About half of the phosphate present in these preparations is hydrolyzed by mild acid with loss of the coenzymatic activity. The hydrolysis constant for the first phosphate at 37°C. in 0.25 *N* acid was found to be 3.1×10^{-4} (Table II). The hydrolysis of glucose-1-phosphate is about four times faster under the same conditions ($K = 1.29 \times 10^{-3}$) (5).

TABLE I

Results of the Purification of the Coenzyme

Step	Amount of extract	Micromoles of coenzyme	Relation P total/P acid-labile
1. Filtered yeast extract	1700 ml.	650[a]	226[b]
2. After Pb and H_2S	1300 ml.	680[a]	130[b]
3. Alkali, Mg^{++}, Pb, H_2S	580 ml.	—	15[b]
4a. First Ba salt	5.06 g.	545[a] 680	10
4b. Fractionated Ba salt	1.85 g.	450	6.5
5a. Acetone precipitation	—	F_1 50	2.2
		F_2 128	2.1
		F_3 107	2.5
		F_4 28	3.8
5b. Final Ba salt (F_1+F_2)	150 mg.	140	2.07

[a] Estimations by enzymatic method.

[b] P total/μM of coenzyme.

The fact that the value of *K* remained constant up to 65% hydrolysis shows that the preparation was not appreciably contaminated with other labile esters.

The connection between coenzymatic activity and acid-labile phosphate was revealed during the last stages of the purification where both values were always parallel. This parallelism was also evident during the acid hydrolysis (Table II).

Heating in 0.1 *N* acid at 100°C. leads to complete liberation of the labile phosphate in 9–10 min. Fig. 2 shows the course of the hydrolysis compared with that of glucose-1-phosphate.

After it was proved that the labile phosphate is related to the activity it became necessary to investigate the nature of the rest of the molecule.

TABLE II

Hydrolysis of the Coenzyme in 0.25 N HCl at 37°C.

$$K = \frac{1}{t_2 - t_1} \log_{10} \frac{100 - x_1}{100 - x_2}$$

Time	Liberation of P inorg. Per cent of acid-labile	$K \times 10^4$	Decrease in coenzymatic activity
min.			*per cent*
60	3.50	2.67	—
180	11.4	3.08	0
360	22.0	3.11	23
540	32.5	3.50	37
720	39.7	2.78	34
1440	64.5	3.25	77
		Mean: 3.06	

The intact coenzyme gives a barium salt which is insoluble in water, but after hydrolysis most of the part bearing the second phosphate becomes water soluble. This indicates that the labile and stable phosphate belong to the same molecule.

The estimation of aldose by titration with hypoiodite revealed that after mild acid hydrolysis one equivalent appeared per molecule of phosphate split off. None was reactive in the intact molecule.

These facts indicated that the second phosphate belonged to an aldose phosphate, and the enzymatic detection of glucose-6-phosphate was attempted. The procedure was based on the fact that muscle

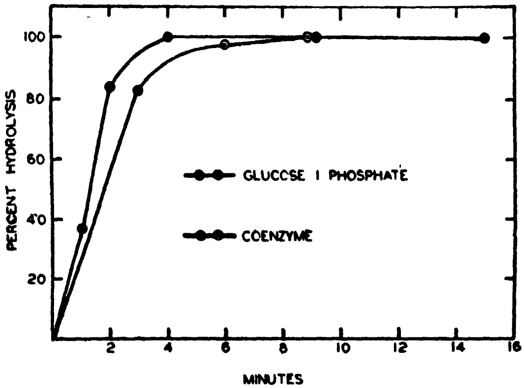

Fig. 2. Hydrolysis of the labile phosphate of the coenzyme and of glucose-1-phosphate in 0.1 N H_2SO_4 at 100°C.

extracts contain enzymes which catalyze the equilibrum glucose-1-phosphate ⇄ glucose-6-phosphate ⇄ fructose-6-phosphate. Using aged and fairly dilute extracts, it is unlikely that other reactions will interfere. Moreover, since fructose can be easily estimated, this affords a method for detecting any one of the 3 esters. The coenzyme before and after hydrolysis was incubated with the extract, and fructose was then estimated. As shown in Table III, the expected amount of fructose was formed from the hydrolyzed coenzyme, and none from the nonhydrolyzed.

TABLE III

Formation of Fructose Phosphate from the Hydrolyzed Coenzyme

(details in text)

Incubation for 20 min. at 37°C. of 0.1 ml. of enzyme solution plus additions. Total volume 0.5 ml.

Additions	Colorimeter readings
6.2 *μM* of coenzyme	12
6.2 *μM* of coenzyme hydrolyzed	128
2 *μM* glucose-1-phosphate	45
5 *μM* glucose-1-phosphate	98
Enzyme alone	8

The correct identification of glucose-6-phosphate with relatively small amounts of material is a problem which has not been worked out satisfactorily. Two experiments were carried out in which polarimeter readings were taken before and after hydrolysis. The values obtained before hydrolysis gave $[\alpha]_D = +63°$ to $+70°$ in acid solution and calculated for an hexose diphosphoric acid. After hydrolysis, $[\alpha]_D = +30°$ to $+32°$ for an hexose monophosphoric acid. For glucose-6-phosphoric acid, the corresponding value is $+34.2°$ to $+35.1°$ (6,7).

The solution was then separated into water-soluble and insoluble barium salts. The soluble fraction gave $[\alpha]_D = +12°$ to $+15°$, calculated for the barium salt of an hexose monophosphate. The value given by Robison and King (6) for glucose-6-phosphate is $+16.6°$.

Since these results were not considered conclusive, additional information was sought in the acid and alkaline hydrolysis and by preparing the osazone. The results were as follows:

	Soluble Ba salt from hydrolysis of coenzyme	Glucose-6-phosphate
	per cent	*per cent*
Hydrolysis, 1 *N* acid at 100°C., 1 hr.	0–1	1.8 (6)
Hydrolysis, 1 *N* acid at 100°C., 2 hr.	4–5	3.5 (6)
Hydrolysis, 0.2 *N* alkali at 100°C., 3 min.	54	60 (10)

The osazone was undistinguishable microscopically from that of glucose-6-phosphate, and its melting point was 150–153°C. The value given by Robison and King (6) being 154°C.

If it is admitted that glucose-6-phosphate is formed by hydrolysis of the coenzyme, then in the intact substance the position of the labile phosphate can be fixed in 1 because of its facile hydrolysis with acid and because it masks the reducing group. The coenzyme would be, therefore, glucose-1,6-diphosphate, and its high dextrorotation, which decreases on hydrolysis, indicates the α-anomer.

The presence of an aldose component in fructose diphosphate preparations has been discussed by Neuberg, Lustig and Rothenberg (26). Due to the small proportion of the glucose ester, it may have escaped detection by chemical methods or it may have been removed in the highly purified preparations used by Neuberg *et al.*

The synthesis of glucose diphosphate was attempted by treating 1,6-dibromotriacetylglucose with Ag_3PO_4, using the procedure of Cori, Colowick and Cori (5), which proved successful for the synthesis of glucose-1-phosphate. While an active preparation was obtained, thus adding more evidence to the proposed structure, the yield was so low, and purification so difficult, that the method was abandoned. The synthesis starting with glucose-1-phosphate and phosphorus oxychloride was unsuccessful.

All the facts which have been mentioned agree with the proposed structure of the coenzyme, but, since analytically pure preparations have not been obtained, other methods of synthesis are being investigated.

The structure of the coenzyme is important from the point of view of the mechanism of action of the enzyme. Meyerhof *et al.* (11) had found that during the reaction there was no interchange between the hexose phosphates and radioactive inorganic phosphate. Schlamowitz and Greenberg (12) found that labeled glucose did not interchange with the phosphorylated glucose, and postulated that a 1,6-glucose monophosphate was formed as an intermediary in the reaction.

A simple explanation for the mechanism, is that the enzyme would catalyze the transfer of the phosphate-1 of the coenzyme to position 6 of glucose-1-phosphate. The reaction products would thus be glucose-6-phosphate and glucose-1,6-diphosphate. In the reaction the coenzyme would be regenerated at the expense of the substrate.

Another intriguing point has been settled. Cori, Colowick and Cori (13) had found that rabbit muscle phosphoglucomutase could be electrodialyzed without losing activity. They could not reproduce the activation which Kendal and Stickland (3) had observed on adding fructose diphosphate preparations. Moreover, phosphoglucomutase has been purified by Schlamowitz and Greenberg (14), and crystallized by Najjar (15), and the necessity of a coenzyme was not noticed.

Since the coenzyme was found to activate yeast phosphoglucomutase but hardly at all that of rabbit muscle, it seemed possible that the mechanism of action of the two enzymes might be different. However, it has been found that, if the muscle enzyme is tested in the presence of cysteine, as described by Najjar (15), then the addition of coenzyme produces a great increase in activity (Fig. 4, left).

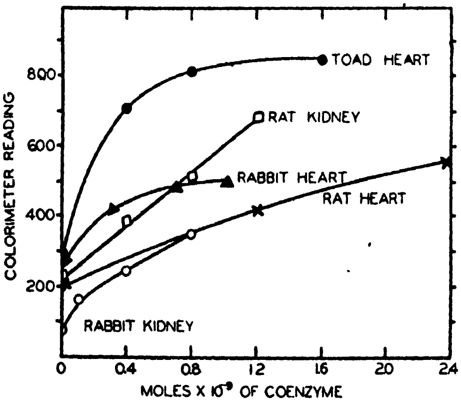

FIG. 3. The activation of phosphoglucomutase from different organs by different amounts of coenzyme. Measurement by the copper reduction method. No cysteine used.

If the muscle phosphoglucomutase is treated with acid in $(NH_4)_2SO_4$ solution as described by Warburg and Christian (16), for the reversible splitting of flavoproteins, then the activity without coenzyme becomes negligible (Fig. 4, right).

In extracts of heart and kidney the effect of the coenzyme can be observed even without the addition of cysteine (Fig. 3).

The coenzyme has been found in every animal tissue thus far investigated (Table IV), and it can be concluded that it is present wherever phosphoglucomutase is present.

EXPERIMENTAL

Enzymatic Estimation of the Coenzyme

Dried brewer's yeast was suspended in 3 vol. of water, incubated 2 hr. at 37°C., and then centrifuged. The supernatant was then diluted with 4 vol. of water. The activity was found to be unchanged on storage in the frozen state for several months. This crude extract was found to be activated by the coenzyme as much as dialyzed or $(NH_4)_2SO_4$-purified preparations.

The measurements were carried out in 12 × 100 mm. test tubes graduated to 7.5 ml. The reaction mixture contained 2 μM of sodium or potassium glucose-1-phosphate 1 μM $MgSO_4$, 0.02 ml. of enzyme solution and coenzyme solution. Total volume 0.3 ml. The reaction was started by adding the enzyme and, after 10 min. at 37°C., it was interrupted by addition of 1.5 ml. of Somogyi's sugar reagent (17).

The tubes were heated 10 min. in a boiling water bath, and then cooled. After addition of 1.5 ml. of Nelson's arsenomolybdic reagent (18) and water to complete the volume the color was measured with a Klett photocolorimeter fitted with filter 52. The values obtained with increasing amounts of coenzyme are shown in Fig. 1.

Since no buffer was used, all the samples were adjusted to pH 7.4–7.5. Unknown solutions were compared with a standard coenzyme, blanks without glucose phosphate were run at the same time. These blanks are necessary because crude solutions may reduce initially or may develop reducing power during incubation. For instance, samples containing saccharose give a high blank, due to the invertase present in the yeast extract. If blanks are high, it is convenient to purify partially by precipitation with lead acetate.

The most frequent cause of error is the presence of salts which inhibit the enzyme, as was observed by Cori, Colowick and Cori (13). Sometimes a precipitation with lead acetate is useful in this case also.

Substances with SH groups hardly affect the activity with the yeast preparations and, moreover, they can be destroyed by heating in alkaline solution.

Usually, 2 or 3 different amounts of the unknown, were compared with a curve obtained in the same series using 2 or 3 samples of the standard solution. The error amounted to about 10%.

The Isolation of the Coenzyme

1. Preparation of the Yeast Extract. The technique of Neuberg and Lustig for preparing fructose diphosphate (4) was used with fresh baker's yeast, saccharose, and ether. The coenzyme content increased during incubation at 30°C. up to 24 hr., and slowly decreased thereafter:

Hours	0	2	5	24
Coenzyme content (μM/l.)	11	185	415	445

The substitution of saccharose for glucose or starch did not improve the yield.

After incubation, the proteins were coagulated by heating, and the mixture filtered through fluted paper.

2. Precipitation with Lead Acetate. To the filtrate of step 1, 2 ml.-% of glacial acetic acid were added, and then excess of 25% lead acetate. The suspension was filtered on

Buchner funnels using "Celite super cell." The cake was suspended in water and decomposed with H_2S. After filtration and aeration, the liquid was made alkaline to phenolphthalein with NaOH.

Comments. The treatment with H_2S should be carried out rapidly, since the coenzyme is acid-labile. The omission of step 2 did not give good results.

3. Destruction of Fructose Diphosphate. The liquid from step 2 was treated with 0.05 vol. of 5 *N* NaOH, and then heated in a large boiling water bath. Half an hour after the thermometer had reached 90°C., the liquid was cooled. Acetic acid was added to pH 7, and then an excess of magnesium acetate. The mixture was left overnight in the icebox. The $Mg_3(PO_4)_2$ was filtered off and NH_4OH added until phenolphthalein gave a rose color. After filtration, the absence of inorganic phosphate in the filtrate was checked analytically. Excess lead acetate was then added. The suspension, which was slightly alkaline to litmus, was filtered, and the precipitate decomposed with H_2S, filtered and aerated.

Comments. During the alkaline treatment the liquid becomes dark brown, and nearly all the organic phosphate is hydrolyzed. The Seliwanoff reaction for fructose (19) becomes negative. The amount of alkali can be increased up to 1 *N* and the heating up to 3 hr. without appreciable destruction of coenzyme.

The elimination of the inorganic phosphate with magnesia mixture in the usual manner resulted sometimes in a loss of about 40% of the coenzyme by coprecipitation. Excess NH_4OH should be avoided, since it interferes in the subsequent lead precipitation.

4. Barium Fractionation. Excess barium acetate and 0.2 vol. of alcohol were added, and the liquid was filtered using Celite super cel. The dry precipitate (4a, Table I) was suspended in 10 vol. of water, cooled in ice, and adjusted to pH 3.5 (bromo-phenol blue) with HCl. The precipitate was discarded, and the liquid was treated with 3 vol. of 96% ethyl alcohol. The acid barium salt was separated with hardened filter paper, dissolved in water, adjusted with $Ba(OH)_2$ to pH 8, and 0.2 vol. of alcohol was added. The precipitate was then centrifuged and dried with alcohol and ether (4b, Table I).

Comments. This fractionation has been described by McFarlane (20) for the purification of fructose diphosphate. In some cases, the procedure was repeated, and in others the acid barium salt was dried and treated directly as described in 5. The latter procedure is to be preferred.

After this step it can be considered that all the acid-labile phosphate corresponds to the coenzyme since all the fructose diphosphate has been destroyed and glucose-1-phosphate is separated as the barium soluble salt.

5. Precipitation with Acetone. The barium salt was suspended in water as a thick paste, cooled, and 5 *N* H_2SO_4 was added until thymol blue gave a rose color. After checking for the absence of barium ions, the suspension was filtered on a Buchner funnel through Celite and a small amount of Norit. The filtrate was treated with 10 vol. of acetone and centrifuged. The oily precipitate was dissolved in a small volume of water and stored in ice (Fraction 1). To the supernatant a drop of concentrated NH_4OH was added followed by centrifugation. Three or four fractions were collected in this manner and analyzed separately for inorganic, acid-labile and total phosphate (5a, Table I). The fractions which showed a relation total phosphate/acid-labile near 2 were mixed, acidified with acetic acid and treated with $BaCl_2$. The $BaSO_4$ was centrifuged off, and washed. The combined supernatant and washings were adjusted to pH 8 with filtered saturated $Ba(OH)_2$, 0.2 vol. of alcohol added and centrifuged. The precipitate was washed with 20% alcohol until the supernatant gave no reaction for chloride and then dried with alcohol-ether (5b). The yield and purification in different steps are shown in Table I. One of the barium salts obtained in this manner and dried *in vacuo* over $CaCl_2$ had an acid-labile phosphate content of 3.48% and 8.5% of total phosphate. For an anhydrous dibarium hexose diphosphate the theoretical total phosphate is: 10.15%. The Seliwanoff reaction (19) for fructose gave values which were equal to those given by equivalent amounts of glucose. In some preparations, the values were slightly higher but could be lowered by a second treatment with alkali.

Acid Hydrolysis

The hydrolysis was carried out in 0.25 *N* HCl at 37° ± 0.1°C., and both the phosphate split off and the coenzymatic activity were determined. The preparation which had a P total/P acid-labile ratio of 2.4 and contained only about 3% fructose (Roe (19) procedure) was freed from barium by adding the exact amount of H_2SO_4, after centrifuging it was diluted with one volume of 0.5 *N* HCl. It was then immersed in a thermostat, and the samples taken at intervals were pipetted into tubes containing sufficient amount of 0.3 *N* NaOH to neutralize the acid. Both inorganic and acid-labile phosphate were estimated by the method of Fiske and SubbaRow (21). The hydrolysis, which occurs during the development of color is negligible, and no correction was applied for it.

As a check on the whole procedure an experiment was run with glucose-1-phosphate, the value for *K* was found identical to the one given in the literature (5).

For analytical purposes the hydrolysis in 0.1 *N* H_2SO_4 at 100°C. was also studied. The results in Fig. 2 show that after 9–10 min. the phosphate liberated reaches a constant value.

Aldose Estimation

The method of Macleod and Robison (22) was used, and a solution of the sodium salt of the coenzyme. Hydrolysis was carried out in 1 *N* HCl at 100°C. during 7 min. In one experiment, 0.71 ml. 0.02 *N* I_2 was used, and the phosphate split off in the same amount of solution was 7.9 μM. Another experiment gave 0.75 ml. iodine for 7.5 μM phosphate. That is the ratio aldose/labile phosphate was 0.9 and 1.0, respectively. No iodine was used by the nonhydrolyzed coenzyme. A check of the method with the same amounts of glucose gave 96% of the theoretical value.

Enzymatic Identification of Glucose-6-Phosphate

An extract of rabbit muscle was prepared by mincing and extracting with 3 vol. of water. It was used after keeping several days in the ice box and the amount of enzyme necessary was determined in a preliminary experiment. One sample of the coenzyme was hydrolyzed in 0.1 *N* acid 10 min. at 100°C., and then neutralized. Another sample was used intact. The tubes were incubated at 37°C., and then fructose was estimated as described by Roe (19). Results appear in Table III, and show that the amount of fructose formed from 6.2 μM of the hydrolyzed coenzyme corresponds to about 6.5 μM of hexose phosphate.

Rotatory Power before and after Hydrolysis

A 2 dm. tube and sodium light were used in all the experiments.

Experiment I. A solution at pH 8 of the sodium salt of a preparation with a ratio total phosphate/labile phosphate = 2.02 was prepared. Concentration estimated by P content was 1.85%, $\alpha = + 1.72°$. Therefore, $[\alpha]_D = + 46.4°$, calculated for $C_6H_{10}O_6\ (Na_2PO_3)_2$.

The solution was made 1 N with HCl. Concentration = 1.18 g-% $\alpha = + 1.50°$. Therefore, $[\alpha]_D = + 63.5°$ calculated for the free acid: $C_6H_{10}O_6\ (H_2PO_3)_2$.

The same solution was heated 10 min. at 100°C. and cooled: $\alpha = + 0.58°$, which gives: $[\alpha]_D = + 32.2°$, calculated for: $C_6H_{11}O_6 \cdot PO_3H_2$. The values found in the literature for glucose-6-phosphoric acid are: $[\alpha]_D = + 35.1°$ (6) and + 34.2° (7).

The liquid was then neutralized, and excess barium acetate was added. The precipitate was centrifuged off and the supernatant precipitated with two volumes of ethyl alcohol. The precipitate was dried, redissolved in water and clarified by centrifugation. $\alpha = + 0.17°$, concentration = 0.586 g-% calculated from phosphate content. This gives $[\alpha]_D = + 15°$ for $C_6H_{11}O_6PO_3Ba$. The value is somewhat lower than that of barium glucose-6-phosphate. Robison and King (6) give $[\alpha]_{5461} = + 19.6°$; from this, $[\alpha]_D = + 16.6°$, ($[\alpha]_D = [\alpha]_{5461}/1.18$) at 0.5% concentration and + 18° at 8.4%. Other values given in the literature are: + 17.9° (9), + 17.4° (8), + 16.6° (7).

Experiment II. A preparation of the barium salt with a ratio total phosphate/labile phosphate = 2.29 was dissolved in 0.07 N HCl. Concentration calculated from the labile phosphate was 1.86%. $\alpha = + 2.66°$. Therefore, $[\alpha]_D = + 71.5°$ for the free acid

The liquid was made 0.2 N with HCl and heated 10 min. at 100°C. Concentration = 1.32%, and $\alpha = + 0.79°$. This gives $[\alpha]_D = + 30.0°$ for an hexose monophosphoric acid.

The solution was then separated into water soluble and insoluble barium salts, as in Exp. I. Both were dried and studied. From 242 μM of starting material, 183 of inorganic phosphate were recovered, 150 of barium soluble ester, and 51 of barium insoluble ester.

The barium soluble fraction gave $[\alpha]_D = +13°$, calculated for a barium hexose monophosphate.

The barium insoluble fraction dissolved in 0.2 N HCl gave $\alpha = + 0.26°$, and concentration was 0.0134 M.

Properties of the Product of Hydrolysis

The water-soluble barium fraction from the previous experiments was heated at 100°C. in 1 $N \cdot H_2SO_4$. The phosphate liberated was 0 in 1 hr., and 4% in 2 hr. In another exp., 1 and 5%, respectively. For glucose-6-phosphate, Robison and King (6) give 1.8 and 3.5%.

Alkaline hydroylsis was carried out in 0.2 N NaOH. Phosphate was estimated with higher acid concentration so as to decrease the interference of silicate (23). Blanks run under identical conditions were subtracted. Robison and MacFarlane (10) give a value of 60% under the same conditions.

The osazone was prepared as described by McCready and Hassid (24); a copious precipitate was formed, which was compared with the osazone of a mixture of glucose-6-phosphate and fructose-6-phosphate. By microscopic examination both samples were undistinguishable.

After recrystallization from alcohol-chloroform the melting point was 150°–153°C. Robison and King (6) give 154°C.

Synthesis from 1,6-Dibromotriacetylglucose

4.5 g. of 1,6-dibromotriacetylglucose (25) were dissolved in 30 ml. of anhydrous benzene, and refluxed 2 hr. with 7 g. of Ag_3PO_4. In other experiments, longer times of heating were tried with no better results. After filtration the benzene was removed under reduced pressure. The solid was then dissolved in 40 ml. methanol plus 1.6 ml. of 5 N HCl. After 15 hr. at room temperature, the solution was neutralized, and excess barium acetate was added. After 2 hr., the precipitate was separated, washed with 20% alcohol and dried. Obtained: 1 g. of a substance containing 0.66 of inorganic P, 0.85 of acid-labile and 0.29 of acid-stable phosphate (μM/mg.). The substance was active as coenzyme, 1 mg. having the same activity as 0.05 μM of the pure coenzyme. Yield calculated from the bromoacetyl compound 0.5%. Four different preparations were carried out varying some details, but the yield was not improved.

Distribution of the Coenzyme in Animal Tissues

The organs from freshly killed animals were weighed, minced, and suspended in 2 vol. of water. The proteins were removed by heating and centrifuging, the liquid was heated 20–30 min. at pH 9, and then adjusted to pH 7.4. The coenzyme was then estimated on suitable dilutions by the yeast phosphoglucomutase test. Results in Table IV.

TABLE IV

Coenzyme Content of Animal Tissues

(μM/g.)

Tissue	Rat	Rabbit	Toad
Liver	0.012–0.014	0.017	0.06
Kidney	0.009–0.0028	0.0025	—
Muscle	0.006–0.024	0.01–0.04	0.017
Brain	0.005–0.011	—	—
Heart	0.0028	0.05–0.09	0.0014

Action of the Coenzyme on the Phosphoglucomutase of Animal Tissues

Many experiments were carried out with the enzyme of animal tissues using essentially the same technique described for the estimation of the coenzyme. The extracts were obtained with 2 vol. of water and then adjusted to pH 7.5. The amount used was 0.005–0.02 ml.

As shown in Fig. 3, a considerable activation was obtained with the phosphoglucomutase of heart and kidney.

With muscle, the results were variable until use was made of the test described by Najjar (15), in which cysteine is used as an activator. The test was carried out as described by him with minor variations.

As shown in Fig. 4 (left), under these conditions, the addition of coenzyme increases the rate of reaction about 300% as compared with cysteine alone.

Still more demonstrative results were obtained when the method of Warburg and Christian (16) was applied. The preparations obtained in this manner showed practically no activity in the absence of coenzyme (Fig. 4, right). The procedure was as follows. A rabbit muscle extract was obtained by mincing and extracting with 2 vol. of ice cold water. After half an hour it was strained through muslin. For the experiment shown in Fig. 4 (left), this extract was diluted 100 times with cold water immediately before use. For the acid splitting: to 2 ml. of this extract plus 0.7 ml. of saturated $(NH_4)_2SO_4$, 3 ml. of 0.1 *N* HCl were added with stirring with the pipette submerged in the liquid. These operations, as well as the centrifugation, were carried out at 0°C. The precipitate was then dissolved in 2 ml. of water and neutralized in the cold. After centrifuging off denatured protein, the preparation was ready for use and could be stored several days in the frozen state without loss of activity. In the experiment shown in Fig. 4 (right), this solution was diluted with 1 vol. of cold water.

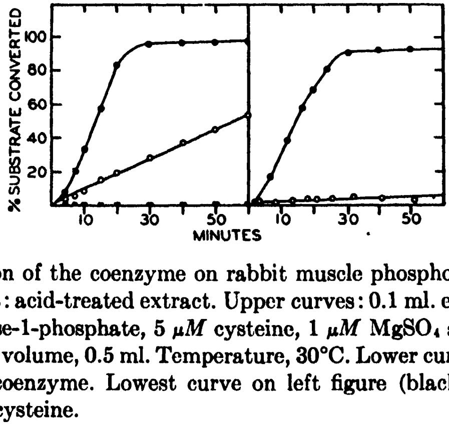

FIG. 4. The action of the coenzyme on rabbit muscle phosphoglucomutase. Left: crude extract. Right: acid-treated extract. Upper curves: 0.1 ml. enzyme solution (see text), 2.3 μM glucose-1-phosphate, 5 μM cysteine, 1 μM $MgSO_4$ and 0.001 μM coenzyme, pH 7.5. Total volume, 0.5 ml. Temperature, 30°C. Lower curves (empty circles): the same without coenzyme. Lowest curve on left figure (black squares) same as upper but without cysteine.

SUMMARY

Methods for the estimation and isolation of the coenzyme of phosphoglucomutase are described. The substance contains two phosphate

groups. The first phosphate can be hydrolyzed by mild acid with the simultaneous liberation of an aldose group (hypoiodite titration).

The hydrolysis constant for the first phosphate in 0.25 *N* acid at 37°C. was found to be 3.1×10^{-4}.

The coenzyme preparations gave a specific rotation $[\alpha]_D = +63°$ to $+70°$ calculated for an hexose diphosphoric ester. On hydrolysis, the dextrorotation decreased to values comparable to those of glucose-6-phosphate. Other properties of the substance remaining after removing the first phosphate, such as behavior toward enzymes, acid and alkaline treatment, and the formation of an osazone, agreed with the properties of glucose-6-phosphate.

An active preparation was obtained by treating 1,6-dibromotriacetylglucose with Ag_3PO_4.

The structure of the coenzyme is, therefore, postulated as α-1,6-glucose diphosphate.

The reversible splitting of the phosphoglucomutase from rabbit muscle is described, as well as the distribution of the coenzyme in some animal tissues.

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The Inhibition of Glucuronidase by Ascorbic Acid and by Heparin [1]

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INTRODUCTION

Fishman and Altman (1) have described a nondialyzable, heat-stable inhibitor of glucuronidase in mammalian serum. It is the purpose of this paper to report some preliminary observations on various inhibitors of glucuronidase.

EXPERIMENTAL

The method of Fishman *et al.* (2) was modified as follows: 1.0 ml. of enzyme solution or tissue homogenate in 0.1 *N* acetate buffer, pH 4.5, and 1.0 ml. of "inhibitor solution" in 0.1 *N* acetate buffer, pH 4.5, were mixed and brought to 38°C. in a water bath. One-half ml. of 0.0025 *M* phenolphthalein glucuronide[2] was added to each experimental tube, but not to the controls, at timed intervals and the contents mixed by whirling. After exactly 60 min. incubation, 2.5 ml. of 0.4 *M* glycine buffer, pH 10.45, was added to each tube to develop the phenolphthalein color, and then 0.5 ml. of 0.0025 *M* phenolphthalein glucuronide was added to each control. After centrifuging off protein precipitates, the amount of phenolphthalein liberated was determined in a Beckmann spectrophotometer set at 555 mμ. All experiments were run in duplicate with one control. Variations between duplicate tubes were less than 3%.

RESULTS

(1) Bovine aqueous humor contains an inhibitor of $(NH_4)_2SO_4$-precipitated ($\frac{1}{2}$ saturation) rat liver glucuronidase prepared according to the method of Fishman (2). This inhibitor is dialyzable and almost entirely destroyed by boiling at pH 4.5 (Table I). Dialysis of the

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[2] Supplied by Drs. Glen Morrow and Herbert Brendler of Brady Urological Institute The Johns Hopkins Hospital, Baltimore.

TABLE I

Effect of Bovine Aqueous Humor on Glucuronidase

	Phenolphthalein γ/*hr.*	Change *per cent*
(1) Aqueous humor amounting to 30% of the incubated fluid	0	—
(2) Liver glucuronidase control[a]	51.0	—
(3) Control + 30% aqueous humor	35.5	−30
(4) Control+30% dialyzed aqueous humor	56.0	+10
(5) Control+30% boiled aqueous humor	47.0	−8

Acceleratory Effect of Dialyzed Aqueous Humor

	Phenolphthalein γ/*9 hr.*	Change *per cent*
(1) Liver glucuronidase control[a]	17.0	—
(2) 30% bovine aqueous (dialyzed)	0.0	—
(3) Control+30% dialyzed aqueous	27.5	+62

[a] Precipitated by ½ saturated $(NH_4)_2SO_4$.

aqueous humor not only removed the inhibitor but revealed the presence of an accelerator for liver glucuronidase activity. This acceleratory effect could be greatly amplified by using smaller amounts of glucuronidase and longer incubator times (Table I). It therefore seemed advisable to investigate a group of compounds found in the aqueous humor and also various related compounds. .

TABLE II

Inhibitory Effect of Ascorbic Acid, Hyaluronic Acid and Heparin

	Phenolphthalein γ/*hr.*	Change *per cent*
(1) Liver glucuronidase control[a]	32.2	—
(2) Control+20 mg.-% ascorbic acid	5.5	−83
(3) Control+2 mg.-% ascorbic acid	25.0	−22
(4) control+0.2 mg.-% ascorbic acid	32.1	0
(5) Control+100 mg.-% hyaluronic acid[b]	16.2	−50
(6) Control+40 mg.-% hyaluronic acid[b]	17.5	−46
(7) Control+4 mg.-% hyaluronic acid[b]	21.3	−34
(8) Control+0.4 mg.-% hyaluronic acid[b]	31.5	−2.4
(9) Control+40 mg.-% heparin sodium	15.2	−52.5
(10) Control+4 mg.-% heparin sodium	16.0	−50
(11) Control+0.4 mg.-%heparin sodium	17.6	−45
(12) Control+0.04 mg.-% heparin sodium	31.2	−3.1
(13) Control+4 mg.-% heparin sodium+ 40 mg.-% hyaluronic acid[b]	16.1	−50

[a] Precipitated by ½ saturated $(NH_4)_2SO_4$.

[b] Impure preparation of potassium hyaluronate.

(2) Ascorbic acid[3] was found to be an effective inhibitor of glucuronidase activity. Aqueous humor concentrations of ascorbic acid reduce enzyme activity by 80–90% and blood concentrations inhibit activity by 20–30%. Representative figures are presented in Table II. Comparable concentrations of glucuose, glucuronic acid, and glutathione did not influence the hydrolysis significantly.

TABLE III

Effect of 40 mg.-% Heparin Sodium on Rat Organ Homogenates[a]

Organ	Weight of tissue homogenized	γ/hr./cc.	γ/mg./hr.	Inhibition
	mg.			*per cent*
Liver	31.6	67.1	21.3	—
Liver + heparin		10.3	3.2	−85
Kidney	80.2	32.5	4.1	—
Kidney + heparin		9.9	1.2	−70
Spleen	36.8	68.6	19.0	—
Spleen + heparin		33.2	9.0	−52
Lung	65.4	27.5	4.2	—
Lung + heparin		12.0	1.8	−56
Submaxillary	178.0	12.4	0.7	—
Submax. + heparin		3.5	0.2	−72
Adrenal	48.4	24.5	5.1	—
Adrenal + heparin		12.8	2.6	−48
Ovary	47.4	46.5	9.8	—
Ovary + heparin		20.7	4.4	−55
Fetus	80.8	27.2	3.4	—
Fetus + heparin		12.7	1.6	−53
Seminal vesicle	191.0	24.9	1.3	—
Sem. ves. + heparin		6.4	0.3	−74
Choroid plexus	10.6	2.6	2.5	—
Chor. plex. + heparin		0.9	0.8	−65

[a] The tissue was weighed, homogenized in 10 cc. $N/10$ acetate buffer and 1 cc. of the homogenate was used for each determination.

(3) Heparin sodium[4] is an inhibitor of liver glucuronidase, producing a maximum inhibition of about 50% at concentrations above 0.4 mg.-% heparin. The inhibition is not increased above about 50% by 100-fold

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[3] Merck U. S. P.

[4] Supplied by Abbott Laboratories, N. Chicago, Ill., in powdered form without preservatives.

TABLE IV

Effect of Testes Extracts on Heparin and Hylauronic Acid Inhibition

	Phenolphthalein γ/*hr.*	Change *per cent*
(1) Liver glucuronidase control[a, b]	30.0	—
(2) Control+2 mg.-% testes extract[b]	29.9	0
(3) Control+4 mg.-% hyaluronic acid[b, d]	18.7	−37.5
(4) Control+4 mg.-% hyaluronic acid[b, d]+ 2 mg.-% testes extract	29.3	−2
(5) Control+0.7 mg.-% heparin sodium[b]	14.8	−51
(6) Control+0.7 mg.-% heparin sodium+ 2 mg.-% testes extract[b]	15.4	−48.5
(7) Liver glucuronidase control[a, c]	30.9	—
(8) Control+testes extract 100 mg.-%[c]	31.5	+2
(9) Control+heparin sodium 0.4 mg.-%[c]	17.0	−45
(10) Control+heparin sodium 0.4 mg.-%+ testes extract 100 mg.-%[c]	30.0	−3

[a] Precipitated by ½ saturated $(NH_4)_2SO_4$.

[b] Incubated 1 hr. at 38°C. before glucuronidase and substrate added.

[c] Incubated 20 hr. at 38°C. before glucuronidase and substrate added.

[d] Impure preparation of potassium hyaluronate.

increases in the heparin concentration (Table II). Impure samples of hyaluronic acid[5] also inhibit up to 50% at concentrations of 40 mg.-% and above. Furthermore, the hyaluronic acid preparation and heparin are not additive to above 50% inhibition (Table II). Although heparin does not inhibit $(NH_4)_2SO_4$-precipitated glucuronidase more than 50%, it does inhibit homogenates of rat liver, kidney, submaxillary gland, and seminal vesicle 70–85% (Table III).

(4) The hyaluronic acid inhibition was destroyed by 1 hr. previous incubation with testes preparations[6] containing about 9 viscosity-

TABLE V

Acceleratory Effect of Testes Extract on Glucuronidase Activity

	Phenolphthalein γ/*hr.*	Change *per cent*
(1) Liver glucuronidase control[a, b]	26.4	—
(2) Control+testes extract 400 mg.-%[b]	38.6	+46
(3) Testes extract 400 mg.-%[b]	0	—

[a] Precipitated by ½ saturated $(NH_4)_2SO_4$.

[b] Incubated 18 hr. at 38°C. before substrate added.

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[5] Supplied by Schering Corp., Bloomfield, N. J.

[6] Supplied by Schering Corp., Bloomfield, N. J.

reducing units of hyaluronidase/mg. The heparin effect required 20 hr. incubation and about 100 times as much testes extract for complete destruction (Table IV). Although the testes preparation had no ability to hydrolyze phenolphthalein glucuronide, when it was incubated for 18 hr. at 38°C. with liver glucuronidase, an acceleratory effect of 50% or more was obtained (Table V).

Epinephrine, mandelic acid and lactate had no remarkable effect on glucuronidase activity.

DISCUSSION

The ability of ascorbic acid to inhibit glucuronidase is definite and marked. The mechanism of this inhibition will require further investigation. The aqueous humor's high content of ascorbic acid accounts for at least part of its inhibitory properites.

The inhibition by heparin is remarkable in that it is limited to 50% inhibition over more than 100-fold concentration change when $(NH_4)_2SO_4$-precipitated enzymes are used. With tissue homogenates, however, some organs are inhibited to as much as 85%. This strongly suggests that we are dealing with at least two glucuronidases, only one of which is inhibited by heparin. Separation and study of these fractions would be of great interest. The two glucuronidases described by G. T. Mills (3) precipitated by 35% and 50% saturated $(NH_4)_2SO_4$ are both inhibited only up to about 50% heparin. In our experience the portion of liver glucuronidase which is most readily inhibited by heparin is found in that portion of liver homogenate which is precipitated by 0.5 hr. incubation at pH 4.5 (Table VI). The effect of the hyaluronic acid

TABLE VI

Heparin Inhibition of Glucuronidase Activity in Various Fractions of Liver Homogenate

	γ	*per cent*
Solution A + buffer	37.5	—
Solution A + heparin 40 mg.-%	13.5	−64.0
Solution B + buffer	47.5	—
Solution B + heparin 40 mg.-%	23.4	−50.5

Solution A: Protein ppt. of liver homogenate after incubation at 38°C. for 30 min. at pH 4.5, resuspended.

Solution B: Supernatant of A pptd. with 75% saturated $(NH_4)_2SO_4$. ppt. resuspended.

Solution C: Supernatant from B after dialyzing away $(NH_4)_2SO_4$. *No activity.*

(Total activity of Solution B = 7 times Solution A) preparation used appears to be similar to that of heparin. It may be due to the hyaluronic acid itself or perhaps due to some sulfonated mucoid impurities.

Histochemically, using the method previously described (4), heparin has an inhibitory effect on all rat tissue sections, but it is most marked on liver, kidney, submaxillary gland, seminal vesicle, and bronchial mucous membrane.

The testes preparation may contain a true accelerator but it seems more likely that it accelerates by the removal of an inhibitor. We have demonstrated that the testes extract will remove the inhibition of hyaluronic acid in 1 hr. and of heparin in 20 hr. Furthermore, acceleration of the liver glucuronidase is only evident after preliminary incubation with the testes extract.

Histochemically, rat cornea was found to contain no glucuronidase activity but, if the incubation is done in the presence of testes extract, activity is quite marked. This may possibly be explained on the basis of the enzymatic removal by the testes preparation of an inhibitor, possibly the hyaluronosulfate of the cornea. Further work on this is in progress. The acceleratory properties of the dialyzed aqueous may be related to the acceleration produced by testes extracts, and is also more marked after prolonged incubation. The possibility that tissue extracts, in which the glucuronidase activity was enhanced by incubation with testes extracts and with dialyzed aqueous humor, might contain a heparin-like endogenous inhibitor, led us to try the effect of adding protamine to the enzyme preparations. Very slight increase in activity was obtained with the addition of protamine in concentrations ranging from 0.004 to 40 mg.-%.

SUMMARY

(1) Glucuronidase activity of liver is inhibited by bovine aqueous humor.

(2) Ascorbic acid reduces glucuronidase activity by as much as 90%.

(3) Heparin and impure hyaluronic acid preparations inhibit $(NH_4)_2SO_4$-precipitated glucuronidase up to 50%.

(4) The failure of over 100-fold increase in heparin concentrations to further inhibit past 50%, and the fact that homogenates of the same organ are inhibited up to 85%, suggests the presence of at least two glucuronidases.

(5) Testes preparations have an acceleratory effect on glucuronidase, perhaps by removing an inhibitor.

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The Effect of Steroid Hormones on the Fatty Liver Induced in Rats by Dietary Means

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INTRODUCTION

It has been recognized that endocrine factors play a part in the complex mechanism which regulates the amount of fat stored in the liver. Involvement of the female sex hormones was demonstrated in studies from these laboratories. Estrone administered orally to intact or castrated female rats fed an alipotropic diet acted as a lipotropic agent (1). Ovariectomy favored the development of fatty livers, while castration in the male was without demonstrable effect (2). The present communication includes the results of a more extended study of the effect of steroid hormones having various types of physiological activity on the development of fatty livers in male and female rats receiving a diet deficient in lipotropic factors or containing methionine as lipotropic agent.

EXPERIMENTAL

These experiments were carried out independently in the Cleveland and Philadelphia laboratories. The same general procedures were followed as were previously reported (1).

Young adult rats, weighing 150 g. or more, were used. All of the animals in the Cleveland laboratory and the majority in the Philadelphia laboratory were of the Sprague-Dawley strain. Some of the rats used in Philadelphia were albinos obtained from a local dealer.

Control and treated animals were run simultaneously in all experiments. When all of the animals of an experiment could not be run at one time, equal numbers of the various groups were started together. All of the animals received the same alipotropic diet, which was of low protein-high fat type. It, and the vitamin supplements, are described in the previous paper (1). In general, the animals were allowed to eat *ad libitum*. In the experiments with ethinyl estradiol, however, it was found that the hormone reduced the appetite of the rats to such an extent that it was necessary to run pair-fed controls.

Six crystalline hormones[1] were used: estrone, estradiol benzoate, ethinyl estradiol, testosterone, progesterone, and desoxycorticosterone acetate.[2] All were given orally, the daily dose being dissolved in 2 drops of cottonseed oil. The oil solution was dropped directly into the mouth of the animal and was taken readily. Any variation in the daily dose due to this method of administration was negligible in comparison with the amount of hormone required to produce a demonstrable lipotropic effect. When several amounts of the same hormone were given, the levels of dosage were varied by several hundred per cent. Control animals were given 2 drops of oil daily. The only exception to this procedure was with testosterone at the highest level fed (5 mg./day). This amount of testosterone could not be dissolved in a convenient quantity of oil, so ethylene glycol was substituted, the control animals receiving an equal amount of the solvent alone.

When methionine was given, the daily dose (50 mg. DL-methionine),[3] was dissolved in 1 ml. of water, and mixed with the vitamin B supplement. In certain of the experiments, where a number of the animals did not drink this mixture, it was given to all by stomach tube.

At the end of the 21 day experimental period, the animlas were sacrificed and the liver analyzed for total lipide with the same technique used in the previous experiments.

Results

The principal data have been assembled in the tables. The level of liver fat found in the control groups may vary considerably from one experiment to another without apparent variations in experimental procedure. Thus, each group must be compared with its own control group run at the same time. For this reason, the data from the two laboratories (those from the Philadelphia laboratory in Tables I and II, those from the Cleveland laboratory in Table III) have been tabulated separately; corresponding Roman numerals have been used for comparable experiments. Similarly, each experiment has been so arranged that each treated group may readily be compared with its appropriate control group.

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[1] Ethinyl estradiol and desoxycorticosterone acetate were kindly supplied by Roche-Organon, Inc.

[2] Designated in the tables as Doca.

[3] Kindly furnished by Wyeth, Inc.

Estrone, which had already been extensively tested with female rats, was used only with the males in this series. Alone, it showed no appreciable lipotropic effect (Table I, Expt. Ia). When it was used as an adjunct to methionine (Table I, Expt. IIa), the liver fat was 12.3%, while, with methionine alone, it was 16.7%. This difference is suggestive only, being without statistical significance.[4]

When estradiol benzoate was used in amount molecularly equivalent to the estrone in both male and female rats (Table I, Expt. Ia and Table II, Expt. IIIc) the hormone alone showed no definite lipotropic effect, although in each case the treated group had a slightly lower

TABLE I

The Effect of Hormones on the Development of Fatty Livers in Male Rats[a]

Experiment	No. of rats	Treatment	Rat weight		Food intake	Liver	
			Initial	Change		Weight	Total lipide
			g.	*per cent*	*g./day*	*g.*	*per cent*
I-a	12	Control	162 (150–180)	−10.2 ±1.5	6.5 ±0.30	7.7 ±0.39	17.6 ±1.8[b]
	10	Progesterone 600 γ/day	160 (150–186)	− 8.6 ±2.6	6.9 ±0.32	8.9 ±0.82	19.4 ±2.3
	12	Doca 275 γ/day	163 (152–170)	−12.2 ±2.5	6.4 ±0.34	7.4 ±0.28	16.1 ±1.9
	13	Testosterone 60 γ/day	163 (147–190)	−10.0 ±1.6	6.7 ±0.34	8.1 ±0.41	20.2 ±1.8
	11	Estrone 30 γ/day	167 (154–212)	−9.6 ±2.2	6.5 ±0.30	7.6 ±0.65	16.9 ±2.4
	11	Estradiol benzoate 40 γ/day	163 (150–182)	−13.1 ±1.0	6.1 ±0.21	7.3 ±0.41	14.8 ±2.3
-b	10	Control	162 (145–176)	−13.4 ±1.4	5.9 ±0.17	7.2 ±0.31	22.0 ±2.1
	10	Testosterone 5 mg./day	163 (150–176)	−13.3 ±1.2	5.2 ±0.13	8.4 ±0.41	25.8 ±2.5
-c	10	Control	163 (155–170)	− 6.2 ±2.0	7.1 ±0.27	6.9 ±0.50	23.1 ±2.5
	9	Control[c]	162 (150–183)	−18.1 ±0.8	5.0 ±0.25	5.9 ±0.42	19.7 ±2.7
	12	Ethinyl estradiol[c] 30 γ/day	164 (150–180)	−21.1 ±1.3	4.9 ±0.19	5.6 ±0.24	8.0 ±1.0

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[4] A difference of means greater than 2.5 times the standard error of the difference of the means, is generally accepted as significant; this corresponds to a P value equal to or less than 0.01.

TABLE I—*Continued*

Experiment	No. of rats	Treatment	Rat weight		Food intake	Liver	
			Initial	Change		Weight	Total lipide
			g.	*per cent*	*g./day*	*g.*	*per cent*
II-a	10	Methionine 50 mg./day	160 (151–173)	−5.0±3.0	6.9±0.25	6.6±0.47	16.7±2.9
	10	Methionine+progesterone 600 γ/day	165 (151–187)	−3.4±1.3	7.4±0.20	6.7±0.51	16.2±2.5
	10	Methionine+doca 275 γ/day	164 (152–172)	−3 2±1.6	7.1±0.29	6.6±0.26	13.6±1 9
	11	Methionine+testosterone 600γ/day	164 (152–181)	−4.5±1.6	7.2±0.28	6.7±0.21	13.7±1.8
	9	Methionine+estrone 30 γ/day	162 (155–177)	−8.8±1.6	6.8±0.20	6.1±0.27	12.3±1.8
	10	Methionine+estradiol benzoate 40 γ/day	166 (155–180)	−6.8±2.2	7.0±0.19	6.4±0.28	10.5±0.6
-b	9	Methionine[c] 50 mg./day	165 (153–189)	−14 4±1.5	4.9±0.11	5.7±0.24	8.3±0.9
	9	Methionine+ethinyl estradiol[c] 30 γ/day	167 (152–185)	−18.4±1.9	4.6±0.15	6.1±0.28	5.6±0.32

[a] Philadelphia laboratory.

[b] Standard error of the mean.

[c] In these experiments the control groups were pair-fed with those receiving ethinyl estradiol.

liver fat than the controls. When the hormone was used with methionine (Table I, Expt. IIa and Table II, Expt. IVa), a markedly lower figure than with methionine was noted, 10.5% instead of 16.7% in the case of the males, 7.2 as compared with 12.5 for the females. These differences stood up well under statistical analysis.

Ethinyl estradiol (Table I, Expt. Ic and Table II, Expt. IIId) was found to have a very high lipotropic activity. Since food intake was low in animals receiving ethinyl estradiol, the control animals were pair-fed with the treated ones to eliminate this as a contributing factor to any observed lipotropic effect. The marked curtailment of food intake and consequent weight loss did not prevent the development of typical fatty livers in the control groups, the males having an average value of 19.7% liver fat, the females, 15.4%. The values were somewhat lower than those of control animals allowed to eat *ad libitum:* males, 23.1%; females, 22.6%. Animals receiving ethinyl estradiol had liver fat values about half those of their controls, the values being 8.0% for the males and 9.0% for the females.

Limitation of food intake did cause marked reduction of liver fat in methionine-fed groups, but the effect of ethinyl estradiol was still plain when animals receiving the same amount of food were compared. In the group of males receiving methionine (Table I, Expt. IIa and b) the animals eating *ad libitum* had an average liver fat of 16.7%, those on restricted food intake, the pair-fed group, 8.3%; ethinyl estradiol given with the methionine brought the value still lower, to 5.6%. The females showed a similar picture (Table II, Expt. IVa and b: methionine-treated animals eating *ad libitum,* 12.5%, methionine-treated with limited food intake, 6.9%, and those receiving methionine plus ethinyl estradiol, 5.2%. The effect of ethinyl estradiol on appetite was more

TABLE II

The Effect of Hormones on the Development of Fatty Livers in Female Rats[a]

Experiment	No. of rats	Treatment	Rat weight		Food intake	Liver	
			Initial	Change		Weight	Total lipide
			g.	*per cent*	*g./day*	*g.*	*per cent*
III-a	10	Control	162 (151–190)	−10.3 ±1 2	6.3 ±0.17	8.5 ±0.95	29.8 ±3.1[b]
	9	Progesterone 600 γ/day	158 (150–180)	−11.4 ±1 6	6.4 ±0.17	7.8 ±0.71	28.4 ±3 8
-b	10	Control	159 (150–169)	−8.4 ±1.4	6.3 ±0.35	6.4 ±0.48	23.4 ±3.1
	10	Progesterone 600 γ/day	160 (152–166)	−6.6 ±1.8	6.5 ±0.22	6.8 ±0.48	22.9 ±2.6
-c	13	Control	159 (150–180)	−11.2 ±1.7	5.8 ±0.20	7.7 ±0.54	20.5 ±2.3
	7	Doca 275 γ/day	159 (152–171)	−11.1 ±2.6	5.7 ±0.25	6.9 ±0.59	17.6 ±1.9
	11	Testosterone 60 γ/day	161 (151–180)	−13.7 ±1.6	5.2 ±0.16	7.6 ±0.53	21.5 ±2.4
	11	Testosterone 200 γ/day	159 (150–176)	−9.7 ±2.6	5.7 ±0.19	7.8 ±0.37	20.4 ±2.6
	10	Estradiol benzoate 40 γ/day	160 (149–187)	−12.4 ±1.9	5.6 ±0.32	7.3 ±0.45	18.2 ±2.9
-d	10	Control	158 (147–185)	−10.6 ±1.2	6.4 ±0.23	7.3 ±0.42	22.6 ±2.7
	9	Control[c]	153 (140–187)	−18.6 ±1.7	4.7 ±0.14	5.2 ±0.26	15.4 ±1.5
	13	Ethinyl estradiol[c] 30 γ/day	154 (138–189)	−18.8 ±1.4	4.7 ±0.20	6.5 ±0.26	9.0 ±1.2

TABLE II—*Continued*

Experiment	No. of rats	Treatment	Rat weight		Food intake	Liver	
			Initial	Change		Weight	Total lipide
			g.	*per cent*	*g./day*	*g.*	*per cent*
IV-a	11	Methionine 50 mg./day	160 (155–167)	−3.1 ±2.4	6.4 ±0.27	6.2 ±0.32	12.5 ±1.6
	10	Methionine +progesterone 600 γ/day	166 (154–185)	−1.5 ±1.7	7.1 ±0.17	6.4 ±0.22	10.1 ±0.67
	10	Methionine +doca 275 γ/day	159 (153–164)	−.12 ±1.8	6.7 ±0.34	6.4 ±0.32	12.0 ±1.4
	10	Methionine +testosterone 600 γ/day	161 (153–171)	−1.8 ±1.5	7.2 ±0.18	6.6 ±0.34	12.1 ±1.2
	10	Methionine +estradiol benzoate 40 γ/day	161 (152–170)	−7.0 ±1.5	5.8 ±0.22	5.7 ±0.20	7.2 ±0.39
-b	8	Methionine[c] 50 mg./day	171 (150–221)	−18.5 ±1.5	3.9 ±0.10	4.9 ±0.30	6.9 ±0.39
	8	Methionine +ethinyl estradiol 30 γ/day	177 (152–246)	−21.5 ±1.6	3.9 ±0.14	6.6 ±0.61	5.2 ±0.32
-c	7	Methionine[c] 50 mg./day	166 (159–177)	−11.7 ±2.1	4.3 ±0.28	5.5 ±0.36	8.1 ±1.0
	7	Methionine +ethinyl estradiol 5 γ/day	165 (155–171)	−16.6 ±2.5	4.2 ±0.25	5.7 ±0.16	5.0 ±0.17

[a] Philadelphia laboratory.

[b] Standard error of the mean.

[c] In these experiments the control groups were pair fed with those receiving ethinyl estradiol.

marked in this group of females than in any of the groups which had been observed previously. Several animals did not eat at all and had to be eliminated. A second group receiving only 5 γ of the hormone daily, one-sixth of the dose previously given, was run (Table II, Expt. IVc). In this group, food intake was increased somewhat and weight loss was less than in the previous one. Even with this small amount of hormone a normal liver fat was found, 5.0%, as compared with 8.1% for the paired control group.

In all of these experiments with ethinyl extradiol and methionine the results show a singificant effect of the hormone. With the males, the change from 8.0 ± 0.9% to 5.6 ± 0.32% is 2.8 times the standard error of the difference of the means, a difference as definite from a mathematical standpoint as the more obvious decrease from 16.7 ± 2.9% in the animals receiving methionine with unlimited food intake to 8.3 ± 0.9% in the restricted group, where the number of standard errors between

TABLE III

The Effect of Hormones on the Development of Fatty Liver in Male and Female Rats[a]

Experiment	Sex	No. of rats	Treatment	Rat weight		Food intake	Liver	
				Initial	Change		Weight	Total lipide
				g.	*per cent*	*g./day*	*g.*	*per cent*
I	M	15	Control	316 261-362	−6.5 ±0.94	9.4 ±1.0	14.2 ±0.58	11.7 ±1.9[b]
		10	Progesterone 600 γ/day	315 268-372	0.7 ±1.9	10.4 ±0.7	16.1 ±1.1	12.7 ±1.1
		10	Doca 100 γ/day	305 259-322	−6.9 ±2.2	9.7 ±0.61	15.1 ±0.57	12.0 ±1.3
		10	Testosterone 600 γ/day	298 260-328	−2.4 ±2.1	10.2 ±0.43	13.9 ±0.79	14.0 ±2.1
II	M	10	Methionine 50 mg./day	320 273-366	−2.0 ±1.8	10.7 ±0.34	15.1 ±0.57	9.3 ±0.66
		10	Methionine + progesterone 600 γ/day	342 284-402	−2.1 ±1.2	10.2 ±0.55	15.9 ±0.83	10.0 ±0.82
		10	Methionine + doca 100 γ/day	319 273-380	−1.7 ±2.1	10.8 ±0.44	16.6 ±0.59	10.2 ±1.2
		10	Methionine + testosterone 600 γ/day	314 288-340	−3.1 ±1.7	11.4 ±0.32	15.4 ±0.44	12.2 ±1.7
III	F	15	Control	192 178-226	−0.8 ±1.3	7.5 ±0.77	10.8 ±0.45	19.1 ±1.8
		10	Progesterone 600 γ/day	218 176-234	1.3 ±1.1	7.5 ±0.47	10.5 ±0.36	9.3 ±0.46
		10	Doca 100 γ/day	196 185-232	3.6 ±1.9	8.1 ±0.83	9.8 ±0.67	11.7 ±0.82
		10	Testosterone 600 γ/day	192 180-200	9.1 ±1.2	8.9 ±0.28	11.6 ±0.43	19.9 ±2.6
IV	F	10	Methionine	202 188-237	3.7 ±1.7	8.1 ±0.27	10.0 ±0.51	8.2 ±0.41
		10	Methionine + progesterone 600 γ/day	211 195-234	1.4 ±1.8	7.1 ±0.43	11.1 ±0.41	7.5 ±0.42
		10	Methionine + doca 100 γ/day	196 180-223	3.6 ±1.9	8.1 ±0.83	9.8 ±0.31	7.0 ±0.33
		10	Methionine + testosterone 600 γ/day	189 181-203	5.6 ±1.1	8.7 ±0.45	10.5 ±0.36	11.4 ±0.99

[a] Cleveland laboratory.

[b] Standard error of the mean.

the means is also 2.8. Considering the two experiments with females, the difference between 6.9 ± 0.39% and 5.2 ± 0.32% liver fat is 3.4 times the standrad error of the difference of the means, that between 8.0 ± 1.0% and 5.0 ± 0.17%, 3.0 times the standard error. Looking at the results from another angle, of the 15 control animals in the two studies with females considered together, the lowest value found was 5.3% liver fat, while 8 of the animals had a fat content higher than 7.0%. In the group of 15 hormone-treated animals the highest value found was 7.0% and there were 8 animals with a liver fat of less than 5.0%. Even with balanced feeding the hormone-treated animals lost, in general, a little more weight than their controls. Considered mathematically, the differences are not significant. They were also much smaller than the differences in wieght loss which occurred when liver fat was lowered by limitation of food intake. The largest difference was in the second series of female rats with methionine, where the average loss of weight in the control group receiving only methionine was 11.7 ± 2.1% and that of the animals treated with ethinyl estradiol and methionine, 16.6 ± 2.5%. The values for the error of the mean indicate that there was considerable spread of the values and overlapping of the groups. In the liver fat values in this particular experiment there was no overlapping, the lowest value in the control group being 6.1%, while the highest value of the treated group was 5.8%.

The non-estrogenic hormones were tested at much higher levels than the estrogens, preliminary tests having indicated that small doses were quite ineffective. Even at the highest levels fed: progesterone, 600 γ/day, desoxycorticosterone acetate, 275 γ/day and testosterone, 600 γ/day to females and 5 mg./day to males, no specific or consistent differences could be found. In the Philadelphia groups, there were no differences of mathematical significance. In the Cleveland series, two groups did show significant deviation. These were females receiving progesterone and desoxycotricosterone acetate (Table III, Expt. III) which had liver fats much lower than their controls. These results were not reflected in the females receiving methionine, nor in the males with or without methionine.

In several of the experiments the estrus cycle of the female rats was followed to see how it was affected by these orally administered hormones. Animals receiving estradiol benzoate were in constant estrus as had been the rats given estrone in earlier studies. This was generally true also for rats given ethinyl estradiol, but in some cases there was irregularity in the estrus. Since there can be no question of the absorption of the ethinyl estradiol which is the estrogen best adapted to oral administration, this result may probably be attributed to inhibitory action of ethinyl estradiol on the anteroir pituitary, while the action of estrone and estradiol benzoate is limited to the target organ. About half of the rats receiving 600 γ/day of testosterone, the highest amount given to female animals, were never in estrus after the first few days of the experiment. The rest showed suppression but not complete elimination of the estrus cycle. Progesterone and desoxycorticosterone acetate had no effect on the estrus.

Discussion

The participation of estrogenic hormones in control of liver has been clearly demonstrated. This activity does not negate the importance of choline or methionine as lipotropic agents. Rather, the greater activity of the hormone when used with methionine suggests that its function may be to induce more efficient use of methionine as a methylating agent. It has been observed (3) that, whereas choline added to an alipotropic diet will give normal liver fat values, methionine rarely does so, though it will prevent the appearance of more than a slight excess of fat in the liver.

It is well recognized that fatty livers are less likely to develop where there is inanition and weight loss. Since estrogenic hormones tend to produce these conditions, the possibility that they might be responsible for the observed lipotropic effects was considered. Only with ethinyl estradiol were these factors of appreciable magnitude and here they were carefully controlled by paired feeding. The analysis of the data given above shows that there were significant reductions in liver fat when animals receiving ethinyl estradiol were compared with control animals receiving the same amount of food. Although decrease in food consumption may well be responsible for part of the activity of the estrogenic hormones, particularly when they are given in conjunction with a moderate amount of methionine, the data of this study as well as those of the previous report (1) indicate that this cannot be the complete explanation of the lipotropic effect found.

As explained in the earlier report (1), the suggestion that estrone might show lipotropic action was based on the observation that estrone pellets implanted in the spleen of rats on a cirrhosis-producing diet decreased somewhat the severity of the cirrhosis. It seemed most likely that such pellets acted directly on the liver. For this reason the hormones in the liver fat studies were administered orally on the assumption that, being absorbed largely through the liver, they might exert an effect on it. Ethinyl estradiol largely escapes loss of its estrogenic activity when given orally, and its effect on appetite and body weight discussed in the preceding paragraph give further evidence of its activity in the body. Extrone and estradiol benzoate lose a large part of their estrogenic activity when given by mouth; however, under the conditions of our experiments, a sufficient amount escaped destruction in the liver to keep the animal in constant estrus. This admits the possibility that the estrogens may well exert their lipotropic action through an extrahepatic mechanism. Ethinyl estradiol, the most effective of the estrogens tried, is noted not only for its high order of potency as an estrogen but for its profound systemic effects when given orally in very small doses. This problem will receive further consideration in another communication (4).

The question arose as to whether the lipotropic activity of the estrogenic hormones might be caused by a protein-sparing, particularly a methionine-sparing, action. The anabolic activity of estrogens, though less than that of androgens, is appreciable (5). In experiments dealing with anabolic acitvity the hormones are generally given subcutaneously but, as has been shown, even with oral administration there was considerable active hormone present in the body. The amounts used in our experiments were, however, very much smaller than those required to show nitrogen retention, which are of the order of 1 mg. per day or more even for androgens. The uniformly negative results with testosterone also make such an explanation unlikely. If such a mechanism were effective, testosterone, provided it escaped destruction in the liver, should show activity similar to that of the estrogens. Testosterone, in amounts comparable with those of the estrogens, having been found completely ineffective, the dose was increased 20-fold to 600 γ/day. At this level testosterone was exerting sufficient systemic effect to suppress in large measure the normal estrus cycle of the female rats. Even when 5 mg. per day was given to male rats there was no lipotropic effect.

Using progesterone and desoxycorticosterone acetate, the results from the two laboratories were not entirely consistent. As pointed out earlier, one experiment in females with each of these hormones indicated a lipotropic activity when it was used alone, but not when it was used with methionine. In view of this discrepancy, the experiments were repeated on other groups of animals with only negative results. Any lipotropic action of these two hormones must depend on some very precise condition of the animals or the experimental regime. Certainly there is no consistent lipotropic activity of progesterone or desoxycorticosterone comparable with that of the estrogenic hormones.

SUMMARY

Three estrogenic hormones, estrone, estradiol benzoate, and ethinyl estradiol, showed lipotropic activity when 30 γ/day was administered orally to rats fed a diet conducive to fatty livers.

Ethinyl estradiol was the most active of the hormones, effecting marked reduction of liver fat in both male and female animals, whether or not methionine was given. With estrone and estradiol benzoate in male rats, the effect of the hormone alone was small, but given with 50 mg. per day of methionine it enhanced considerably the lipotropic effect of the methionine. The same result was found with estradiol benzoate in female rats and a similar effect of estrone in females has been reported previously. Since the decreased food intake observed chiefly with ethinyl estradiol would, in itself, cause decrease in liver fat, this factor was carefully controlled in the experiments with this hormone. There was definite further lipotropic activity not explainable on this basis. It is suggested that the hormone may allow more efficient use of methionine as a lipotropic agent.

Progesterone, desoxycorticosterone acetate and testosterone did not show this lipotropic effect even when given in much larger amount than the estrogenic hormones.

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The Effect of Glycerine and Aniline on Tobacco Mosaic Virus Infectivity [1,2]

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INTRODUCTION

The experiments herein described were carried out in an attempt to discover the reason for the conflicting results on the effect of a mixture of glycerine and aniline on the infectivity of tobacco mosaic virus as reported by Lauffer (1) and by Bawden and Pirie (2). While studying the optical properties of tobacco mosaic virus solutions, Lauffer selected a mixture of glycerine and aniline as a solvent for the virus because it had a refractive index approaching that of the virus particle. Using biological infectivity as a measure of the intactness of the virus particle, he ran tests to show that the infectivity was not decreased by 10 minute exposure to a solvent containing 48% glycerine and 50% aniline. He concluded from these results that the virus protein particle was not denatured or destroyed by such treatment. When Bawden and Pirie (2) repeated the infectivity experiment, they found that contact of the virus with similar glycerine-aniline mixture caused initial, very rapid reduction in infectivity, followed by relatively little change on prolonged contact.

METHODS

The aniline was redistilled. The fraction boiling at slightly under 180°C. was collected and stored in an amber bottle. The glycerine was dried at 100°C. and stored in an amber bottle. Quantitative measurements of the infectivity of tobacco mosaic virus were made by the method of Price and Spencer (3,4). In those cases in which the significance of an apparent change in infectivity was not obvious upon inspection of the data, the standard deviations of the ratios of the infectivities of the control and tested samples were computed by the method outlined by Price and Spencer (4). The significance of a deviation of the ratio from unity was then evaluated by the

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[2] Contribution No. 7P–48 of the Department of Physics, University of Pittsburgh.

usual procedures of statistical inference. A result was deemed significant if it could have occurred by chance less than one time in 20.

EXPERIMENTAL RESULTS

Results of typical experiments are shown in Table I. When exposed to a solvent containing 50% aniline and 48% glycerine for 10 min., preparation I exhibited a small but statistically insignificant decrease of infectivity as determined by inoculation on *N. glutinosa*. The infectivity of virus preparation II was decreased markedly when treated similary. Additional experiments on these 2 preparations showed that this difference was reproducible. Two other virus preparations were studied, one of which behaved like preparation I and the other of which was unstable, even in solvents containing as little as 15% aniline.

TABLE I

Effect of Glycerine-Aniline Solvents on the Infectivity of Tobacco Mosaic Virus

Virus preparation	I		II	
Conc. of virus in contact with solvent (mg./cc.)	1.1		1	
Conc. of virus in *M*/10 phosphate buffer at time of inoculation (mg./cc. × 10^5)	1.1	0.11	1.0	0.10
No. of half leaves inoculated	24	24	16	16
Average no. of lesions per half leaf	37	6	4	1.6
Average no. of lesions per half leaf for control at same conc.	46	6	24	9

Numerous attempts were made to correlate these differences in stability with other measurable differences in the virus preparations, but no success was achieved. The possible effect of pH on the stability was investigated. Portions of one virus preparation were dispersed for 10 min. periods in solvents containing 15% aniline and 75% glycerine buffered with appropriate *M*/10 buffers to pH values of 9.2, 4.9, and 3.0, respectively. Virus activity was not decreased significantly at pH 9.2 and 4.9. However, it was increased significantly at pH 3.0.

DISCUSSION

It is evident from the results obtained in this study that tobacco mosaic virus may or may not lose infectivity to a significant extent when held for ten minute periods in contact with solvents containing high concentrations of aniline. Two preparations were relatively stable even in solvents containing 50% aniline by volume. This result confirms that reported earlier by Lauffer (1). Two other preparations were relatively unstable, like the preparation described by Bawden and Pirie (2). Attempts to correlate these differences with other measurable differences in the virus preparations failed. The discrepancy in the literature can be accounted for on the basis that some virus preparations behave one way and others behave the other way, but the reason for this difference has not been discovered.

SUMMARY

Four different tobacco mosaic virus preparations were dispersed for 10 min. periods in solvents containing varying amounts of glycerine and aniline. By subsequent lesion counts it was found that two of the preparations showed significant decreases in infectivity while the other two exhibited no appreciable loss of infectivity, even in solvents with a volume composition of 50% aniline, 48% glycerine, and 2% water.

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The Vitamin A Activity of Certain Carotenoids in the Chick[1]

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INTRODUCTION

Recently it has been observed that rats fed certain stereoisomers of β-carotene accumulated vitamin A in amounts that were proportional to the known capacities of these compounds to promote growth (1). α-Carotene, however, gave rise to only ½ as much vitamin A as that expected from the growth response, while cryptoxanthine was approximately twice as effective for storage as for growth (2). These differences between compounds could not be ascribed solely to differences in absorbability.

The chick was selected for further study because the metabolism of carotenoids in this species seems to differ in several ways from that in the rat. The vitamin A requirement of the chick per kg. of body weight is reported to be approximately 8–10 times that of the rat (3–6). Lutein and zeaxanthine accumulate in certain tissues of chicks fed these substances, whereas only traces appear in the tissues of rats. With has suggested that cryptoxanthine may be more active for the growth of the chick than β-carotene (7), although for equivalent growth in the rat approximately twice as much cryptoxanthine is required as β-carotene (8). With's suggestion was based on the growth responses of chickens fed yellow corn. In the present study α-carotene, β-carotene, cryptoxanthine, and neo-β-carotene B were fed in pure form to chicks on a semi-synthetic diet low in vitamin A, and both the growth responses and the accumulated amounts of vitamin A were determined.

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METHODS

α-Carotene and β-carotene were prepared from a commercial crystalline carotene, neo-β-carotene B was prepared from β-carotene by iodine isomerization, and cryptoxanthine was isolated in pure form from the ripened fruit of *Dyospyros kaki L.* (Japanese persimmons). Methods followed in the preparation of these pure compounds have been described previously (1,2). Vitamin A was fed as halibut oil. The various supplementary compounds were dissolved in cottonseed (Wesson) oil at concentrations such that 3 drops, measured by calibrated droppers, contained the desired daily supplement together with 0.5 mg. of α-tocopherol.

Day old cross-bred (New Hampshire, ♂♂, single comb white leghorn ♀♀) chicks from hens fed Diet B-1 of Robblee *et al.* (9) were housed in groups of 4–6 in electrically heated batteries with raised screen floors. The birds were fed a synthetic diet low in vitamin A (Table I), on which severe deficiency symptoms usually developed in 22–26 days. After 14 days, at which time several chicks exhibited typical deficiency symptoms, these plus several of the heaviest and the lightest birds were discarded and the remaining chicks were divided into groups comparable in weight. One group received no supplement, while another received 100 I. U. of vitamin A daily by dropper and served as the positive control. Each of the remaining groups was given a daily supplement, either of vitamin A or of one of the carotenoids listed in Table II. Supplementation was continued for 18 days and weight changes during this period were recorded.

TABLE I

Diet Low in Vitamin A

Dextrin	600 g.
Casein	180 g.
Gelatin	100 g.
Cottonseed oil[a]	50 g.
Salts[b]	60 g.
Choline HCl[c]	2 g.
Vitamin mixture[d]	5 g.
Folic acid[e]	1 mg.
α-Tocopherol	3 mg.
Vitamin D_3[f]	2400 AOAC chick units

[a] Wesson oil.

[b] $CaCO_3$, 1200g.; K_2HPO_4, 1290 g.; $CaHPO_4$, 1038 g.; $MgSO_4 \cdot 7H_2O$, 604 g.; NaCl, 670 g.; ferric citrate·6 H_2O, 110 g.; KI, 3.2 g.; $MnSO_4 \cdot 4H_2O$, 31.6 g.; $ZnCl_2$, 1 g.; $CuSO_4 \cdot 5H_2O$, 1.2 g. The Mn, Zn, and Cu salts were ground separately, and the KI was ground separately with NaCl prior to mixing.

[c] Added as an alcohol solution.

[d] A mixture containing: thiamine HCl, 1.2 g.; riboflavin, 1.8 g.; Ca pantothenate, 4 g.; niacin, 10 g., pyridoxine HCl, 1.6 g.; biotin, 0.06 g.; 2-methyl-1, 4-naphthoquinone, 0.1 g.; cystine, 600 g.; inositol, 200 g.; glucose, 181.2 g.

[e] Folic acid was added to the diet in H_2O solution prior to the inclusion of gelatin.

[f] Vitamin D_3 (saponified) added in ethanol solution.

In another growth experiment (Table II, Series III) the chicks were depleted of vitamin A for only 10 days, at which time deficiency symptoms appeared in several birds. The desired daily dose of carotenoid or of vitamin A and 0.5 mg. of α-tocopherol were added as an ether solution directly to 18 g. of ration and the ration fed *ad lib.* This amount of food was estimated to be the average consumed per chick daily during the 18-day period of supplementation. Fresh ration was prepared every 4–5 days, and frequent analyses showed little destruction of the carotene or vitamin A in the ration.

TABLE II

Growth of Chicks Receiving Various Carotenoids

Daily supplement	No. of chicks	Av. wt. at 2 wks.	Av. wt. gained in 18 days	No. of chicks showing			
				Ataxia	Eye inflammation	Death	
		g.	g.				
Series I							
2.5 γ β-Carotene	8	130	170±31[a]	5	1	2	
5.0 γ β-Carotene	8	136	232±26	4	0	0	
2.4 γ Cryptoxanthine	8	129	148±31	5	3	3	
5.0 γ α-Carotene	8	130	173±39	5	1	0	
10 γ α-Carotene	8	130	243±23	4	0	0	
1.5 γ Vitamin A	8	129	165±38	2	3	0	
3.0 γ Vitamin A	8	130	224±37	2	3	0	
Negative control	8	133		8	4	8	
Positive control	8	133	268±24	0	0	0	
Series II							
2.5 γ β-Carotene	8	129	190±21	4	2	0	
5.0 γ β-Carotene	8	130	222±34	3	2	0	
5.0 γ Cryptoxanthine	8	129	205±21	3	1	0	
Negative control	4	130		4	2	4	
Positive control	4	130	235±40	0	0	0	
Series III							Food consumption g./day/chick
5 γ β-Carotene/18 g. ration	10	103[b]	112±25	7	—	1	20
10γ β-Carotene/18 g. ration	10	98[b]	178±36	4	—	0	23
10γ α-Carotene/18 g. ration	10	97[b]	69±33	10	—	3	11
20γ α-Carotene/18 g. ration	10	100[b]	153±29	7	—	1	18
2.5γ Vitamin A/18 g. ration	10	101[b]	125±38	9	—	0	15
5γ Vitamin A/18 g. ration	10	99[b]	200±20	1	—	0	25

[a] Mean deviation.

[b] Av. wt. at 10 days.

Food consumption was measured at the beginning and at the end of the supplementation period.

In experiments on vitamin storage the basic procedures and method of supplementation were similar to those used in the growth experiment. The experiments were performed in series in each of which several of the carotenoids were fed to groups of depleted chicks. Most of the carotenoids were fed at several levels distributed uniformly among the different series. However, because of the shortage of cryptoxanthine and neo-β-carotene B, these were fed at only one level each. The period of supplementation lasted only 15 days. The chicks were decapitated 24 hr. after the last supplement was given, and the organs were removed and analyzed colorimetrically for carotene and viatmin A by procedures described previously (1,10).

RESULTS AND DISCUSSION

Growth Responses and Deficiency Symptoms

Chicks fed only the basal diet rapidly developed severe deficiency symptoms, they lost much weight, and all died before the end of the 18 day "period of supplementation." The earliest and most common symptom of deficiency was leg weakness, shown first by a staggering gait, with the ataxia becoming progressively more severe until the birds were unable to reach the food. When this occurred, weight losses were drastic. Less frequent symptoms of deficiency included inflammation of the eyes, with the appearance of white cheesy plaques; incrustations on the beaks were also sometimes seen in severely deficient birds. Birds receiving supplements of 100 I. U. of vitamin A daily failed to develop any of these symptoms, with an average gain in weight of 268 g. in 18 days. Birds receiving low amounts of the various carotenoids exhibited intermediate growth rates and moderate deficiency symptoms, depending on the compound fed and the dosage.

α-Carotene appeared to be roughly half as effective as β-carotene in the prevention of deficiency symptoms which were essentially the same in groups fed 5 or 10 γ daily of α-carotene as in comparable groups receiving ½ as much of the β-isomer (Table II, Series I). In this series the growth of the groups fed α-carotene was also at least as good as that of comparable groups fed ½ as much β-carotene. When the desired amounts of the carotenoids were incorporated in 18 g. of ration and were fed *ad lib.*, growth by the groups on α-carotene was less than that of groups receiving half as much β-carotene/g. of diet (Table II, Series III) and the abilities of the compounds to minimize deficiency symptoms paralleled the growth responses. However, the intake of food was significantly less by the groups fed α-carotene than those fed ½ as much β-carotene/g. of food, *e. g.*, an average of 18 g./day by the group receiving 20 γ of α-carotene/18 g. of food *vs.* 23 g./day by the group receiving 10 γ of β-carotene/18 g. of food (Table II, Series III). In terms of the amounts of carotene actually ingested, therefore, α-carotene was probably about ½ as effective as β-carotene in this series also.

Cryptoxanthine appeared to be nearly as effective as β-carotene for growth and the prevention of deficiency symptoms in the chick. In Series I (Table II) a group fed 2.4 γ of cryptoxanthine daily was somewhat inferior in both respects to a group receiving 2.5 γ daily of β-carotene; in Series II a group fed 5.0 γ of cryptoxanthine daily was intermediate in response between groups fed 2.5 and 5.0 γ daily, respectively, of β-carotene. Variations within groups were fairly large and, in general, the response of chicks to carotenoids or to vitamin A appeared to be less uniform than the response of rats to similar supplementation. The two series in Table II, however, suggest that cryptoxanthine is roughly ¾ as effective as β-carotene in promoting growth in the chick and not, as postulated by With, more effective than β-carotene (7). Indeed, the relative activities of the two compounds in the chick are not greatly different from those in the rat (8,11).

Vitamin A proved to be more effective than β-carotene in promoting growth in the chick, whether the supplements were given directly by dropper (Table II, Series I) or were incorporated into the ration (Table II, series III). This difference was also apparent in the abilities of the two compounds to prevent deficiency symptoms. While the experiments were not designed primarily to establish the biological equivalence of vitamin A to 0.6 γ of β-carotene in the chick, calculations from the observed growth responses (Table II) indicated that 0.38 γ of vitamin A promoted growth equivalent to that due to 0.6 γ of β-carotene (1 I. U.) when the supplements were given by dropper (Series I) and 0.24 γ of vitamin A = I. U. when the active materials were added to the diet (Series III). In view of the variations encountered within groups and the inequalities in food intake, this divergence in values is not surprising. The results do, however, suggest that the relative biological activities of β-carotene and vitamin A in the chick are not greatly different from the ratio for rats, 1 I. U. = 0.289 γ of vitamin A, observed in a recent collaborative study (12).

Storage of Vitamin A in Chicks Fed Carotenoids

When α-carotene, β-carotene, or vitamin A were fed to depleted chicks, most of the vitamin A in the body was found in the liver; the small intestine seemed to be the next most important site of storage, and measurable amounts of the vitamin also occurred in the combined lungs, spleen, heart and gonads (Table III). In contrast to the rat, in which small amounts of vitamin A first accumulate in the kidney (1), the kidney of the chick appeared to be relatively unimportant for the storage of vitamin A.

TABLE III

Vitamin A Storage in Various Organs of the Chick

Supplement fed[a]	No. of chicks	Tissues	Vitamin A per chick γ
100 γ α-Carotene	4	LSHG[b]	0.5
	2	Intestine[c]	1.5
	18	Kidney	0.5
	18	Liver	12.4
50 γ β-Carotene	4	LSHG	0.6
	2	Intestine	2.1
	27	Kidney	1.2
	27	Liver	30.2
14 γ Vitamin A (50 I. U.)	2	LSHG	1.7
	2	Intestine	0.9
	23	Kidney	0.2
	23	Liver	4.3

[a] Supplements were fed for 15 days.

[b] Lung, spleen, heart, and gonads.

[c] Determinations were made on entire small intestine.

The amounts of vitamin A in the livers of chicks fed the various carotenoids are listed in Table IV, according to the experimental series. The experiments extended through several seasons of the year and the series occasionally differed from one another in both the growth rate during the depletion period and in the time necessary for depletion, *e. g.*, in Series III the time of depletion ranged from 23 to 27 days as compared to Series VI, in which the time of depletion ranged from 14 to 19 days. Nevertheless, the amounts of vitamin A in the livers of chicks fed a given carotenoid in several series were fairly similar from one series to the next, *e. g.*, on 50 γ of β-carotene daily the amounts of vitamin A found were 43.8, 24.8, and 24.9 γ in Series II, IV, and VI,

TABLE IV

Vitamin A in Livers of Chicks Fed Carotenoids

Supplement	Series	No. of chicks	Depletion time	Weight beginning supplement	Weight gain	Liver	
						Vitamin A	Pigment[b]
			days	*g.*	*g.*	γ	γ
25 γ β-Carotene	III	7	25.2	141	163	8.5	5.9
	V	7	25	134	183	16.2	6.5
50 γ β-Carotene	II	8	24	125	172	43.8	5.7
	IV	11	24	129	143	24.8	6.2
	VI	9	18.3	103	147	24.9	8.8
100 γ β-Carotene	I	12	16[a]	118	111	(82.5)	6.3
	IV	9	23.3	138	149	143	7.8
	V	7	23.4	135	177	168	8.7
100 γ Neo-β-carotene B	IV	9	23.6	137	164	36.1	12.6
100 γ α-Carotene	II	10	25	110	155	12.1	6.3
	V	8	25	138	173	12.8	8.3
200 γ α-Carotene	III	6	24.5	138	168	62.1	10.1
	V	7	26	150	165	77.8	9.5
7 γ Vitamin A	III	7	25.3	144	156	1.2	3.4
	V	8	25.3	129	164	2.9	5.3
14 γ Vitamin A	II	8	24.5	138	163	4.7	4.9
	IV	9	24.5	134	158	4.0	6.5
28 γ Vitamin A	I	12	14[a]	117	119	(46.0)	3.9
	IV	9	23.3	136	161	16.9	5.3
50 γ Cryptoxanthine	VI	9	17.3	97	164	24.3	7.6

[a] All started at this time.

[b] Ether-soluble yellow calculated as carotene. Much of this pigment is noncarotenoid in value.

respectively. In Series I, on the other hand, depletion was not complete when supplementation was begun arbitrarily after 14–16 days on the low A diet, and the amounts of vitamin A found in the liver are not, therefore, directly comparable to those in the other series. When depleted chicks were given 25 γ of all-trans-β-carotene daily for 15 days, an average of 12.3 γ (8.5–16.2) of vitamin A was found in the liver (Table IV); daily doses of 50 and 100 γ of β-carotene in the various series resulted in average stores of 30.2 and 154 γ of vitamin A, respectively. The doses of the other carotenoids fed (Table IV) resulted in the accumulation of intermediate amounts of hepatic vitamin A.

Chicks fed 100 γ daily of neo-β-carotene B accumulated an average of 36.1 γ of hepatic vitamin A. According to the formula used previously in calculating relative potencies (1),

$$\frac{\text{dose of } \beta\text{-carotene}}{\text{storage due to } \beta\text{-carotene}} \times \frac{\text{storage due to other carotenoid}}{\text{dose of other carotenoid}} \times 100,$$

this amount corresponds to activities of 25–72% that of all-*trans*-β-carotene based on stores of 143 γ and 24.8 γ of vitamin A in chicks of the same series fed 100 γ and 50 γ daily of the all-*trans* isomer, respectively. The variations are due to the fact that vitamin A storage does not vary arithmetically with carotenoid intake and, hence, the formula is only applicable to groups in which vitamin A storage is nearly equal. It is probable, therefore, that the biological activity of neo-β-carotene B with respect to β-carotene in the chick is not greatly different from that in the rat, 53% for growth (13) and 48% for storage (1).

Depleted chicks fed 50γ of cryptoxanthine daily for 15 days contained 24.3 ± 3.1 γ of vitamin A per liver as compared with 24.9 ± 7.1 γ in a comparable group fed 50 γ of β-carotene daily (Table IV). On this basis cryptoxanthine is 98% as active as β-carotene, a percentage also reached when the storage of the group fed 50 γ of cryptoxanthine daily is compared with that of chicks fed 25γ of β-carotene. Cryptoxanthine has also been observed to equal β-carotene for the storage of vitamin A in the rat (2).

α-Carotene, on the other hand, appeared to be less than half as effective as β-carotene for the production of hepatic vitamin A in the chick. Chicks fed 200 γ of α-carotene daily accumulated 70.5 γ of vitamin A in the liver as compared to 154 γ in chicks fed only 100 γ of β-carotene daily, and 30.2 γ in chicks fed 50 γ of the β-isomer. As in the rat (1), therefore, the ingestion of α-carotene by the chick tends to result in lower hepatic stores of vitamin A than one would predict on the basis of the growth-promoting power of the carotenoid.

Depleted chicks fed diluted halibut liver oil stored vitamin A in the liver provided the dose was sufficiently high; a daily dose of 28 γ (100 I. U.) of vitamin A resulted in the accumulation of 16.9 γ of the vitamin in the liver after 2 weeks (Table IV). With lesser dosages, growth was good but storage was low. Thus, in spite of claims to the contrary (14), the young chick seems capable of storing vitamin A that has been fed as such, and on a weight basis vitamin A appears to be approximately equal to β-carotene for the production of liver stores. In this respect the metabolism of the two substances in the chick differs from that in the rat, for, in the latter species, daily doses of 11.2 γ of vitamin A (40 I. U.) resulted in slightly larger stores of hepatic vitamin A than daily doses of 40 γ of β-carotene (15).

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SUMMARY

1. For the growth of depleted chicks and the prevention of symptoms of vitamin A deficiency, vitamin A proved to be somewhat more active than β-carotene, α-carotene was about $\frac{1}{2}$ as active as β-carotene, while cryptoxanthine was intermediate in activity between α- and β-carotene.

2. For the accumulation of vitamin A in the livers of chicks, cryptoxanthine was nearly equal to β-carotene; neo-β-carotene B was somewhat more than half as active as the all-*trans*-isomer, while α-carotene was less than half as active as β-carotene. β-Carotene was almost as active as vitamin A itself. Thus, except for certain minor quantitative differences, the relative activities of the carotenoids in the chick were quite similar to those in the rat.

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Effect of "Vitamin P"-Like Substances on Capillary Resistance in Thrombocytopenic Purpura in Rats

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INTRODUCTION

Since the discovery of Szent-Györgyi and coworkers (1) that lemons contain a substance called "vitamin P," which is effective in decreasing capillary fragility in hemorrhagic disorders, much work has been expended in the search for active materials which will protect capillaries. "Vitamin P" action has usually been ascribed to substances which raise the lowered capillary resistance in "vitamin P"-deficient guinea pigs and many natural materials and synthetic substances have such effects (2–8). Natural flavanoid substances protect animals against the lethal effects of X-irradiation (9–11), presumably due to protection against capillary damage.

Since hesperidin was found active in experimental thrombocytopenic purpura in guinea pigs (12) but vitamin K was inactive in similar tests in rats (13), it seemed that the prevention of thrombocytopenic purpura might be a specific test for substances which protect capillaries in hemorrhagic disorders.

METHODS

Antiplatelet serum was made according to the procedure of Zucker (13). Six preparations were made at different times.[1] The blood platelets from approximately 100 rats were suspended in saline and injected intravenously into 5 rabbits at weekly intervals for 5 weeks. With preparations No. 4, 5, and 6, Thephorin (14) 1 mg./kg., was given at the same time as the platelets to prevent anaphylactic shock. A week after the last injection of platelets, the rabbits were bled, the serum collected, pooled, and preserved in the frozen state.

After preliminary observations that the capillary resistance and platelet counts reached minimal values by the third day, the following assay procedure was adopted:

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[1] We wish to thank Miss Edith Titsworth, Chemotherapy Department, for making the first preparation of antiserum.

Eight Sprague-Dawley rats, 150–250 g., were used in each group. The abdominal skin was shaved with a clipper. On the first day, the anti-serum was given subcutaneously in the back and simultaneously the vitamin P preparations were given orally or intraperitoneally. The "vitamin P" preparations were again administered on the second and on the third day. Platelet counts were made on tail blood diluted with 4.8% sodium citrate. Capillary resistance tests were made on the third day, usually about 6 hr. after the last vitamin P treatment, by Dalldorf's method (15).

Negative pressure from the vacuum line was measured with a vacuum gauge and applied through an 8 mm. glass tube to the vaselined skin. Suction was applied for 10 sec. to various areas of the skin and the minimum negative pressure which produced petechiae was taken as the end point. This was estimated only within 50 mm. Hg negative pressure. Since the vacuum line had a maximum negative pressure of 500 mm. Hg, the rats which did not show petechiae at this pressure were given an arbitrary value of 600 mm. Hg. The standard errors and "t" values were calculated according to Fisher (16).[2]

EXPERIMENTAL

Among some 300 normal untreated rats only an occasional one showed petechiae at 500 mm. Hg. The average platelet count of 130 normal rats was 670,000/mm.[3]

The degree of fall in platelet count and capillary resistance depended on the strength of the antiserum, as shown by the results on the control rats summarized in the table. A number of soluble "vitamin P" preparations, as well as various compounds not obviously related to "vitamin P" materials, partially prevented the fall in capillary resistance but did not affect the number of platelets. These compounds, therefore, differentiated the effect of the antiserum on platelets and capillaries. Prophylactic effects on capillary resistance were observed with small doses of "vitamin P" preparations, sodium hesperidin chalcone and disodium salt of 3,4-dihydroxy-4-methoxychalcone-4′-phosphate, with large doses of rutin, hesperidin methylchalcone and epimerized *d*-catechin but not with naringin and neohesperidin.

Epinephrine and adrenochrome were tested because of the claim of the French workers (6,7) that these are the effector agents in protecting capillaries, and that "vitamin P" is an antioxidant for them. In our experiments, epinephrine and adrenochrome had prophylactic effects and there was an additive effect of epinephrine and hesperidin methylchalcone. However, adrenoxyl was inactive.

It has been postulated that "vitamin P" is a sparing substance for vitamin C (1,17). Hiramatsu (12) found that hesperidin was strongly effective against hemorrhages produced by antiplatelet serum, while vitamin C was only slightly effective. In the present experiments, ascorbic acid has no protective action against capillary damage, nor did it increase the effects of hesperidin methylchalcone.

The claim of Skelton and coworkers (18) that large doses of vitamin E increased the lowered capillary resistance and platelet counts produced in dogs by stilbestrol has not been confirmed (19). We found that α-tocopherol phosphate was inactive in preventing the fall in capillary resistance produced by antiserum.

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[2] A "t" value greater than 2.365 (p = .05) was considered to indicate a significant difference between the means.

TABLE I

Effect of "Vitamin P"-Like Materials on Capillary Resistance and Platelet Counts

Treatment					Capillary resistance			Platelet count	
Drug	Dose	Route	Antiserum preparation		Controls average ±S. E.	Treated average ±S. E.	"t"	Controls	Treated
			No.	Dose					
	mg./kg.			*cc./100 g.*	*mm. Hg*	*mm. Hg*		*thousands/ mm.*^{3}	*thousands/ mm.*^{3}
Sodium hesperidin chalcone	3	I. P.	6	0.1	210±19	320±65	1.64	93	56
	6	I. P.	5	1	194±27	450±46	4.78	387	325
	12	I. P.	5	1.25	181±23	444±61	3.90	37	73
	25	I. P.	4	0.2	225±42	450±66	3.54	434	381
	50	I. P.	4	0.2	150±12	470±54	5.70	230	172
	100	I. P.	4	0.2	170±9	475±54	5.40	125	108
	200	I. P.	4	0.2	180±13	525±38	8.70	242	100
Disodium salt of 3,4′-dihydroxy-4-methoxychalcone-4′-phosphate	3	I. P.	6	0.1	170±11	175±23	0.11	140	120
	6	I. P.	5	1	207±24	425±65	2.76	234	255
	25	I. P.	5	1	207±24	437±63	2.96	234	276
1-Dihydroxyphenylalanine	3	I. P.	6	0.1	210±19	200±56	0	93	199
	6	I. P.	5	1.25	181±23	388±76	2.99	37	51
	12	I. P.	4	0.2	225±42	270±62	2.06	434	489
	25	I. P.	4	0.2	150±12	431±69	5.12	230	181
	100	I. P.	4	0.2	170±9	550±27	13.40	125	92
	200	I. P.	4	0.2	180±13	560±18	16.7	242	95
N-(β-hydroxyethyl) gentisamide	50	I. P.	5	1	207±24	300±56	1.22	233	216
	100	I. P.	5	1	194±27	325±52	2.52	387	114
Rutin	200	Oral	1	0.1	335±33	435±60	1.61	—	—
	400	Oral	1	0.1	335±33	475±46	2.50	—	—
	800	Oral	1	0.1	335±33	540±38	3.87	—	—
Adrenochrome	5	I. P.	4	0.2	180±13	490±30	9.50	—	—
	5	I. P.	2	0.1	275±50	356±56	2.25	—	—
	10	I. P.	2	0.1	275±50	470±49	5.27	—	—
	10	I. P.	3	0.1	165±11	495±45	13.75	160	175
Adrenochrome semicarbazone (Adrenoxyl)	5	I. P.	4	0.2	150±12	150±12	0	230	389
Epinephrine	0.5	I. P.	2	0.1	275±50	280±62	0.10	—	—
	1.0	I. P.	2	0.1	275±50	485±74	2.92	205	109
Hesperidin methylchalcone Epinephrine	25 0.5	I. P.	3	0.1	200±23	350±49	2.78	195	145

TABLE I (*Continued*)

Treatment					Capillary resistance			Platelet count	
Drug	Dose	Route	Antiserum preparation		Controls average ±S. E.	Treated average ±S. E.	"*t*"	Controls	Treated
			No.	Dose					
	mg./kg.			*cc./100 g*	*mm Hg*	*mm. Hg*		*thousands/mm.³*	*thousands/mm.³*
Hesperidin methylchalcone Epinephrine	50 0.5	I. P.	3	0.1	200±23	475±50	3.45		
Hesperidin methylchalcone Ascorbic acid	50 400	I. P.	1	0.1	335±33	315±21	0		
Polyporic acid	3	I. P.	6	0.1	170±11	240±42	1.63	140	210
	6	I. P.	6	0.1	210±19	440±57	3.91	93	112
	12	I. P.	5	1	207±24	500±38	4.14	234	149
	25	I. P.	5	1	194±27	444±60	3.76	387	118
	50	I. P.	4	0.2	225±42	540±46	5.40	434	386
Hesperidin methylchalcone	50	I. P.	1	0.1	350±46	470±68	1.60	235	290
	100	I. P.	1	0.1	350±46	510±44	2.35	235	180
	100	I. P.	2	0.1	270±44	350±29	2.86	—	—
	200	I. P.	1	0.1	350±46	590±27	3.75	235	310
	200	I. P.	2	0.1	270±44	440±46	5.00	—	—
Epimerized *d*-catechin	50	I. P.	5	1	194±27	194±26	0	387	316
	100	I. P.	4	0.2	180±13	325±72	2.00	—	—
	200	I. P.	1	0.1	310±48	525±31	3.19	320	280
	400	I. P.	1	0.1	310±48	550±27	3.63	320	250
Sodium gentisate	25	I. P.	4	0.2	150±12	206±54	1.14	230	212
	50	I. P.	4	0.2	170±9	435±67	4.15	125	131
	100	I. P.	4	0.2	180±13	300±57	2.00	—	—
	200	I. P.	4	0.2	180±13	410±66	3.50	242	214
	400	I. P.	4	0.2	180±13	560±18	17.0	242	152
Ascorbic acid	400	I. P.	1	0.1	335±33	350±25	0.30	—	—
Naringin	400	Oral	1	0.1	300±31	300±57	0		
Neohesperidin	400	Oral	1	0.1	300±31	310±46	0.15		
dl-α-Tocopherol phosphate Na salt	400	Oral	1	0.1	300±31	305±44	0.10		
3-Hydroxy-2-methyl-1,4-pyrone (Palatone)	100	I. P.	5	1	181±23	188±54	0.11	37	73
Gentisyl alcohol	100	I. P.	5	1	181±23	206±26	0.72	37	64

The finding of Beiler and Martin (20) that "vitamin P" compounds inhibit hyaluronidase, and of Meyer and Ragan (21) that hydroquinones and quinones inhibit hyaluronidase, suggested that the latter compounds might have "vitamin P" activity. We found activity with small doses of dihydroxyphenylalanine and polyporic acid, with large doses of sodium gentisate and N-(β-hydroxyethyl)gentisamide but none with Palatone or gentisyl alcohol.

DISCUSSION

The decrease in capillary resistance produced by antiplatelet serum in rats was partially prevented with doses as small as 6 mg./kg. of substances claimed to have "vitamin P" activity—sodium hesperidin chalcone (22) and disodium salt of 3,4′-dihydroxy-4-methoxychalcone-4′-phosphate (8). Equal activities were found for L-dihydroxyphenylalanine, polyporic acid, epinephrine and adrenochrome. Although we found no activity with adrenoxyl, adrenochrome derivatives have been claimed (23) to raise capillary resistance in guinea pigs and human subjects. Much larger doses of the "vitamin P" materials—epimerized *d*-catechin (6) and rutin (24)—were necesasry to produce protection. Similar activities were found for sodium gentisate and N-(β-hydroxyethyl) gentisamide. Further comparison with the qualitative results obtained in guinea pig feeding experiments are not profitable because of the divergence of opinion concerning the relative activities of various preparations.

It was also found that doses of hesperidin methylchalcone and epinephrine, which were inactive alone, had an additive effect when given together. Ascorbic acid was not active nor did it have an additive effect with hesperidin methylchalcone. Materials which were inactive included naringin, neohesperidin, sodium salt of *dl*-α-tocopherol phosphate, Palatone and gentisyl alcohol.

The fall in capillary resistance in experimental purpura is prevented by a wide variety of substances, including some which have been claimed to have "vitamin P" activity. As the standard errors in the table indicate, the method of measuring capillary resistance is relatively crude. Large variations occur in the sensitivity of various areas of the abdominal skin. A reliable relationship of the dose of the substance to the response was not obtained but undoubtedly large doses of active materials had significant effects. Therefore, the "*t*" test was applied to the result obtained with each dose of a compound in comparison with the control response obtained in the same experiment.

Hiramatsu (12) considered that experimental thrombocytopenic purpura produced by antiplatelet serum in animals corresponded to human thrombopenic purpura which arises from a fall in blood platelets and damage to endothelium. He found that the capillary damage produced by antiplatelet serum was prevented by hesperidin. We have found that the test is not specific for "vitamin P" compounds since a number of hydroxy-substituted compounds and quinones not obviously related to "vitamin P" have this property. Hiramatsu further considered that the vascular purpura produced by anti-endothelial serum in guinea pigs corresponded to the vascular purpura of human subjects which results from damage to blood vessels by toxins. He found that this type of purpura was also prevented by hesperidin. Possibly X-irradiation produces this type of damage in dogs (11). Rutin and epimerized *d*-catechin are effective in both thrombocytopenic purpura and X-irradiation damage. However, hesperidin methylchalcone, sodium gentisate and dihydroxyphenylalanine are active in the former test but not in the latter. The thrombocytopenic purpura test is, therefore, a less specific or less rigorous test of "vitamin P" activity. It is possible that the protective action against capillary damage is a pharmacological effect related to the antioxidant properties of these substances rather than a specific vitamin action. It may be significant that some of these potential quinone-forming compounds also have antihyaluronidase activity (20,21). Epinephrine probably prevents a fall in capillary resistance because of its vasoconstrictor properties on skin vessels. Although most of the "vitamin P"-like compounds are vasodepressors when administered intravenously, Haley *et al.* (25) suggested that the local vasoconstrictor properties of such compounds on capillary beds may indicate peripheral vasoconstrictor activity.

SUMMARY

The decrease in capillary resistance produced by antiplatelet serum in rats is partially prevented by flavanoid materials and other hydroxy substituted compounds and quinones. The test is not specific for "vitamin P"-like materials.

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A Study of Carboxylase in Soybean Sprouts [1]

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INTRODUCTION

It is known that the soybean contains thiamine and that the seed produces CO_2 as it sprouts. Carboxylase was found in pea roots by Horowitz and Heegaard (1), and Ziejlemaker (2) reports that the total cocarboxylase content remained constant during 12 days germination. The present work is an investigation of carboxylase activity in soybean sprouts, employing the Warburg technique to measure the output of CO_2 and employing pyruvic acid as the substrate. Carboxylase activity reached a maximum in the first stage of sprouting, falling off as the root grew. The carboxylase activity could be restored during the growth period by adding cocarboxylase and manganese, which is a metallic ion known to possess the ability of functioning as the metallic component of the enzyme (7), but the activity could not be increased beyond the maximum observed early in sprouting. The total amount remained constant during the 4 day culture but the amount of thiamine pyrophosphate changed parallel to the carboxylase activity.

EXPERIMENTAL

DEMONSTRATION OF CARBOXYLASE IN SOYBEAN SPROUTS

Separation of the Enzyme. One volume of soybean seeds (*Glycina hispida, maxin*)[2] was soaked in 5 vol. of water and incubated at 30°C. for 6 hr. The beans were then placed on filter paper in Petri dishes and set away in the dark. They were bathed in tap water 4 times daily. After germination, the sprouts were ground in a glass mortar in 1/25 *M* phosphate buffer at pH 6 and strained through muslin. Eight g. of seeds yielded 100 ml. of enzyme solution.

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[1] This material was revised for publication by John R. Lowry, Department of Biochemistry and Nutrition, University of Southern California, who is grateful to Dr. Bradley T. Scheer for advice on preparing the manuscript.

[2] These seeds were grown in Manchuria and sent to Japan in 1943.

Measurement of Activity. Warburg-type measurements, employing pyruvic acid as the substrate, were used. The CO_2 absorbed in the medium was released at the end of a run by addition of tartaric acid. One ml. of the enzyme solution and 1.2 ml. of 1/30 *M* phosphate buffer at pH 6 were placed in the main chamber of the vessel while the side arm contained 0.2 ml. 0.75 *M* sodium pyruvate solution also at pH 6. The other side arm contained 0.05 ml. saturated tartaric acid solution. The center well contained 0.4 ml. 5% KOH and a small piece of rolled filter paper.

Determination of Thiamine. Free thiamine was determined as follows: 50 ml. of the enzyme solution was diluted to 80 ml. with distilled water and brought to a pH of 4.2 with HCl. This was immediately centrifuged and 0.3 g. of acid clay was added to the supernatant. Free thiamine was determined by standard procedures (3). The combined thiamine was freed by treatment of 50 ml. of the enzyme solution at pH 4.2 with 0.1 g. of Takadiastase and 2 or 3 drops of toluene was the preservative. This was then incubated 15 hr. at 36°C. Free thiamine was then determined. The difference between the total and free forms represents the combined thiamine and was taken as the amount of cocarboxylase present.

TABLE I

Carboxylase in Soy Bean Sprouts

Growth stage		Weight (10 g. seeds)	Respiration		With pyruvic acid		Thiamine		Acetoin
			$-O_2$	CO_2	$-O_2$	CO_2	Free	Combined	
		g.	*mm.*^{3}	*mm.*^{3}	*mm.*^{3}	*mm.*^{3}	*mg.*	*mg.*	*mg.*
Dried seed		10	22	97	17	193	44	3.8	0.22
Soaked	3 hr.	20	13	18	13	212	27	25	0.65
	6 hr.	22	17	22	23	278	30	23	—
Setting	1 day	23	37	40	28	216	37	10	0.47
	2 days	28	60	57	35	181	40	3	0.29
	3 days	30	100	56	84	140	40	3.8	—
	4 days	35	90	39	53	109	42	0.4	Trace

Acetoin Formation. The estimation of acetoin was carried out by adding 2.5 mg. 1 *M* pyruvate to 25 ml. of the enzyme solution and allowing the mixture to stand 60 min. with stirring every 10 min. Acetoin was determined after Dirscherl (4) as the dimethylglyoxime Ni salt.

Table I and Fig. 1 show the results of some typical runs. As soon as the seed begins to absorb water, phosphorylation of thiamine appears to begin and carboxylase activity increases. Oxygen intake increases with the growth of the sprouts, while carboxylase activity, cocarboxylase content and acetoin formation decrease.

Apocarboxylase in the Soybean Sprouts. Two enzyme solutions were prepared: (a) from beans soaked in water for 6 hr. and (b) from sprouts grown in the dark (25°C.) for 5 days. Cocarboxylase was synthesized by the method of Tauber (5) and Weil-Malherbe (6). In the main chamber of the Warburg vessel 1 ml. of enzyme solution was mixed with 0.2 ml. of a solution containing 80 γ cocarboxylase and 120 γ manganese/ml. and 1 ml. of 1/30 *M* phosphate buffer solution was added (pH 5.8). The side arm contained 0.2 ml. 0.75 *M* pyruvate solution at pH 5.8. Tests were run 60 min. at 30°C.

The enzyme solution derived from beans soaked 6 hr. was not affected by the added cocarboxylase, while that from the 5 day sprouts clearly was, as is shown by

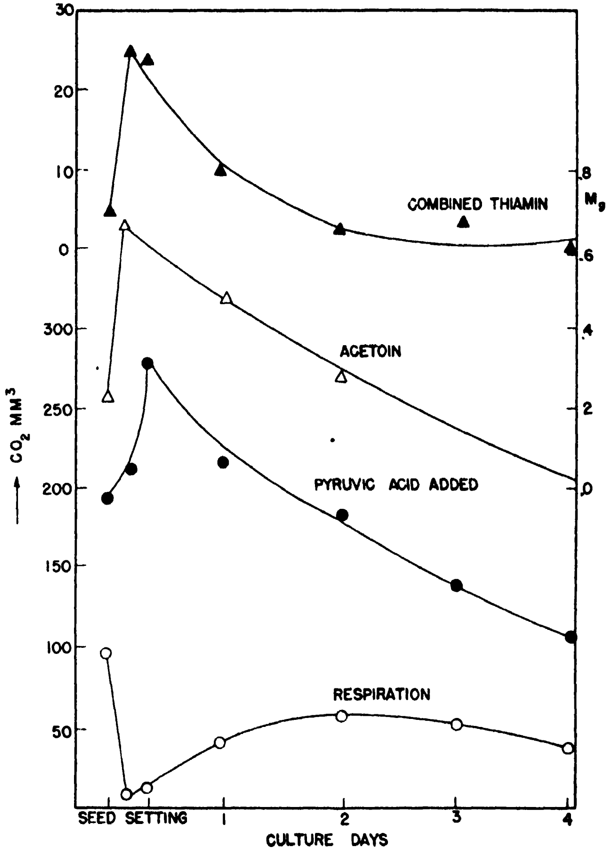

Fig. 1. Carboxylase in soybean sprouts.

Table II. Therefore, it might be supposed that there was a residue of apocarboxylase after the decrease of cocarboxylase in the growing sprouts. However, it was found that oxygen intake increased when cocarboxylase was added to the 5 day enzyme solution, while in the case of the 6 hour enzyme solution no such increase occurred. It is possible that pyruvic oxidase occurs in the sprouts and studies on this enzyme will be reported in a later paper.

TABLE II[a]

Addition Effect of Cocarboxylase to Soy Bean Sprout Carboxylase

In main chamber: 1 ml. of enzyme solution, 0.2 ml. of a solution of cocarboxylase (80 γ of cocarboxylase and 120 γ of manganese/ml.), and 1 ml. of 1/30 *M* phosphate buffer solution (pH 5.8). In side arm: 0.2 ml. of ¾ *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

Enzyme sources	Seed soaked in water for 6 hr. at 30°C.				Sprouts on the fifth day of growth			
Co-carboxylase γ	0		16		0		16	
Manganese γ	0		24		0		24	
Gas volume mm.^{3}	$-O_2$	CO_2	$-O_2$	CO_2	$-O_2$	CO_2	$-O_2$	CO_2
Reaction period, min. 10		61		54		42		46
20		108		102		60		68
30		154		154		79		92
40		191		193		98		115
50		234		231		110		134
60	14	258	16	262	74	127	88	155

[a] The corrections for CO_2-retention were carried out by the addition of tartaric acid to the medium when the experiment was over.

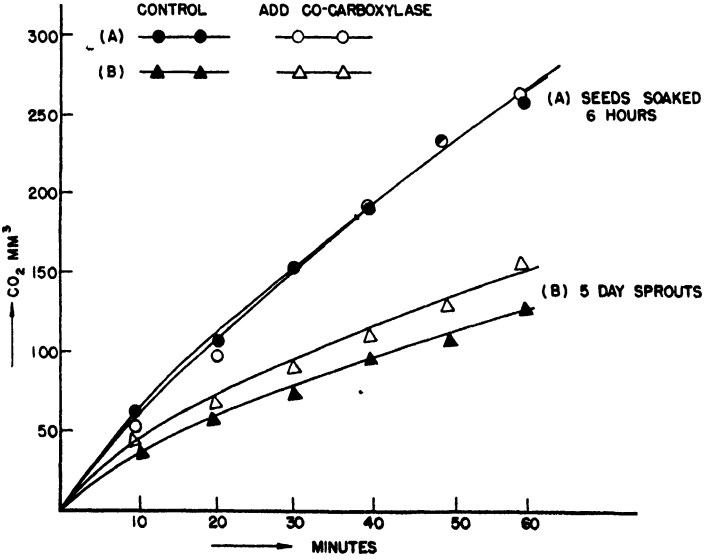

FIG. 2. Addition effect of cocarboxylase.

SEPARATION AND RECONSTRUCTION OF SOYBEAN CARBOXYLASE

Sixteen g. of soybean seeds were soaked in 80 ml. of 4% NaCN solution for 3 hr. at 30 °C. and then crushed in a glass mortar. Phosphate buffer at pH 5.8 (1/25 *M*) was added and the juice pressed out through muslin and made up to 200 ml. This was enzyme solution I. One hundred twenty ml. of this was mixed with 120 ml. of a saturated solution of $(NH_4)_2SO_4$ and 1 part concentrated NH_4OH. This was allowed to stand 1 hr. and then filtered. The precipitate was dissolved in 1/25 *M* phosphate buffer (pH 5.8) and made to 60 ml. This was enzyme solution II. This was put through the same procedure as I and a 30 ml. enzyme solution was obtained (enzyme solution III). Twenty ml. of III was put through the procedure and 15 ml. of enzyme solution IV obtained. Warburg measurements were carried out as previously described. Table III shows the activities of each of the 4 enzyme solutions. Horowitz *et al.* (1) were unable to separate the apoenzyme from the coenzyme using Na_2HPO_4 and alkaline $(NH_4)_2SO_4$ in work with pea roots. Green *et al.* (7), working with yeast carboxylase, were able to affect a dissociation of the enzyme in alkaline systems and studied the factors involved in recombination. In the present work, treatments repeated 4 times failed to effect the separation. If it is assumed that the degree of separation can be given by 1 − (*a*/*b*), the relative separations are given in Table III, wherein *a* and *b* are defined.

TABLE III

Separation of Enzyme into Coferment and Apoferment

In main chamber: 1.0 ml. of enzyme solution, 0.2 ml. of a solution of cocarboxylase (80 γ cocarboxylase and 120 γ Mn) and 1.0 ml. of 1/30 *M* phosphate buffer solution (pH 5.8). In side arm: 0.2 ml. of ½ *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

No. of enzyme solution	Control (a)		Add. cocarboxylase (b)		Relative[a] separation (1-*a*/*b*)
	$-O_2$	CO_2	$-O_2$	CO_2	
	mm.^{3}	mm.^{3}	mm.^{3}	mm.^{3}	
(1)	10	168	12	197	0.15
(2)	15	248	5	239	0.0
(3)	13	125	15	179	0.3
(4)	0	63	2	107	0.41

[a] Where *a* is the value obtained from the isolated apoenzyme and *b* results from apoenzyme plus added cocarboxylase and Mn. This is obviously a measure of the separation achieved.

In another attempt at separation, 16 g. of seeds were soaked in 0.4% NaCN for 4 hr. This yielded 120 ml. of an enzyme solution which was treated with 60 ml. saturated $(NH_4)_2SO_4$ and the mixture placed in the ice chest. Ten ml. of this was filtered and the precipitate dissolved in 1/25 *M* phosphate buffer at pH 5.8 and made to 12 ml. Manometric activities were run on this preparation and yielded 60 mm.^{3} while the control gave 14 mm.^{3} indicating a relative separation of 0.77. Table IV gives more extended results from this preparation.

TABLE IV

Reconstruction of Soy Bean Carboxylase

In main chamber: 1.0 ml. of enzyme solution, 0.2–0.6 ml. of a solution of cocarboxylase and Mn plus 1/30 *M* phosphate buffer solution (pH 5.8) to make a total of 2.0 ml. In side arm: 0.4 ml. of $\frac{1}{5}$ *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

Cocarboxylase solution, ml.[a]		0.0	0.1	0.2	0.4	0.6	
Cocarboxylase, γ		0	8	16	32	48	
Manganese, γ		0	12	24	48	72	
Gas volume, $mm.^3$		CO_2	CO_2	CO_2	CO_2	$-O_2$	CO_2
Reaction period, min.	10	0	8	12	16	2	17
	20	1	13	29	37	3	40
	30	6	25	46	60	5	66
	40	8	32	62	78	5	86
	50	10	39	76	99	5	109
	60	14	48	88	115	5	126
$1-a/b$		0.0	0.71	0.84	0.88		0.89

[a] Amounts taken of a solution containing cocarboxylase and 1/30 *M* phosphate buffer (pH 5.8).

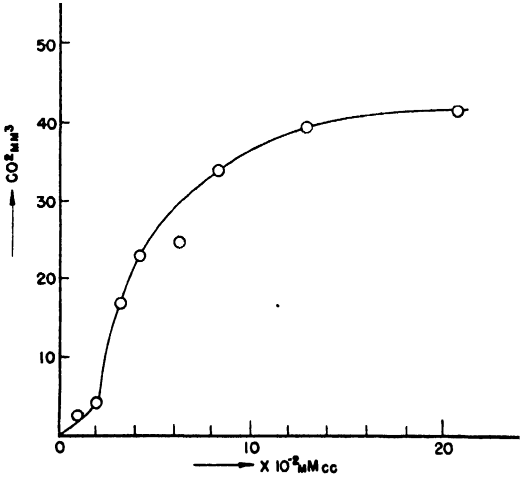

FIG. 3. Effect of substrate concentration.

The General Properties of Soybean Carboxylase

For these studies the same enzyme preparation that yielded the data for Table IV was used. Trial runs indicated that when activities were measured at a pH greater than 6 the medium showed CO_2-retaining power. This was corrected for by adding tartaric acid. Fig. 3 shows the influence of varying amounts of substrate (pyruvic acid)

TABLE V

Influence of Concentration of the Apocarboxylase

In main chamber: 0.2–1.8 ml. of enzyme solution (see text), 0.2 ml. of cocarboxylase mixture sol. and 1/30 *M* phosphate buffer solution (pH 5.8) to make a total of 2.0 ml. In side arm: 0.4 ml. of 0.96 *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

Apocarboxylase, ml. . . Dry weight, mg.... . .	0.0 0.0	0.2 3.08	0.4 6.16	0.6 9.24	0.9 13.8	1.2 18.5	1.5 23.1	1.8 27.3
Produced CO , mm.^{3}	0	27	52	74	94	98	110	107
Produced CO_2, mm.^{3}/ml.	0	11.3	22	31	39	41	46	45

TABLE VI

Influence of Varying Amounts of Cocarboxylase

In main chamber: 1.5 ml. of enzyme solution (see text) containing 24 γ of manganese, 0.0 to 0.50 ml. of cocarboxylase solution (160 γ/ml.) and 1/30 *M* phosphate buffer solution (pH 5.8) to make a total of 2.0 ml. In side arm: 0.4 ml. 1 *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

Cocarboxylase, ml.- . . Cocarboxylase, μ*M*/ml..	0.00 0.000	0.05 0.007	0.10 0.013	0.25 0.033	0.40 0.053	0.50 0.066
Produced CO_2, mm.^{3}	63	94	103	110	115	118
Produced CO_2, mm.^{3}/ml.	26	39	43	46	48	49

TABLE VII

Influence of Manganese Concentration

In main chamber: 1.5 ml. of enzyme solution (see text) containing 80 γ of cocarboxylase (pH 5.8), 0.0 to 0.375 ml. of manganese solution (240 γ/ml.) and 1/30 *M* phosphate buffer solution (pH 5.8) to make a total of 2.0 ml. In side arm: 0.4 ml. of 1 *M* pyruvate solution (pH 5.8). For 60 min. at 30°C.

Manganese, ml.——..... .. Manganese, μ*M*/ml.——.....	0.00 0.00	0.05 0.09	0.10 0.18	0.20 0.36	0.375 0.68	0.00[a] 0.00
Produced CO_2, mm.^{3}	45	115	123	155	156	14
Produced CO_2, mm.^{3}/ml.	19	48	51	65	65	5.8

[a] No added cocarboxylase.

upon the activity. A study of the effect of different apoenzyme concentrations upon activity is shown in Table V. The effect of varying amounts of added cocarboxylase is shown in Fig. 4 and Table VI. The effect of manganese concentration is also shown in Fig. 4 and Table VII. The enzyme solution employed was not fully separated into coenzyme and apoenzyme. However, on the basis of these measurements, one can calculate the proportions of apoenzyme, manganese and cocarboxylase that can nearly restore full activity. The value for the "completely separated" enzyme is 5.8 mm.^{3}/ml. of medium.

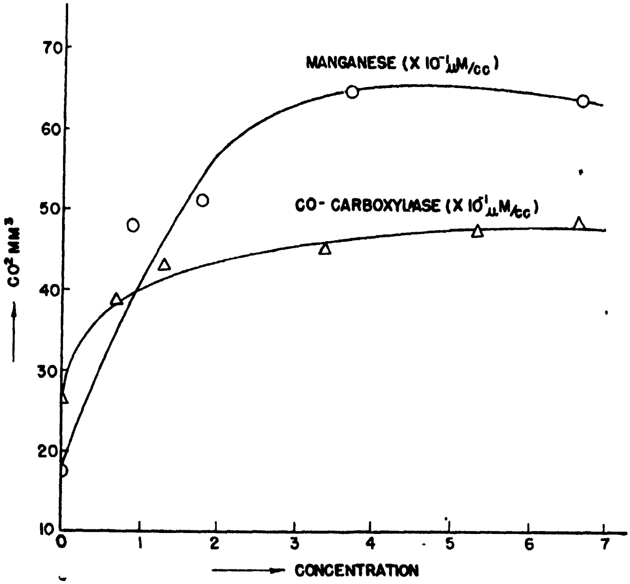

FIG. 4. Influence of the cocarboxylase and manganese concentration.

This value is taken to be *a* in the relation

$$\text{Degree of separation} = 1 - (a/b)$$

To get at a value for *b* one notes from Table VII that the proportions yielding a value of 65 mm.^{3}/ml. medium are 0.16 mm. pyruvate, 23 mg. protein, 33 γ of cocarboxylase and 20 γ of manganese. Thus, the degree of separation is $1 - \frac{5.8}{65}$ or 0.91. The reconstruction experiments on Table IV give a value of 0.89.

It must be emphasized that such calculations cannot be the basis for arriving at the amounts of the various components present in the intact enzyme. It is interesting to calculate the amounts of manganese and cocarboxylase that will "saturate" a given amount of apoenzyme. From Fig. 4 and Table VI it can be seen that 1 ml. of enzyme (23 mg. protein) is "saturated" with 4.3×10^{-2} μM of cocarboxylase and 36×10^{-2} μM of manganese, giving a ratio of cocarboxylase to manganese of 1 to 9.

DISCUSSION

Throughout the present work, manganese has been employed as the metallic ion involved in the carboxylase enzyme complex. This ion is possibly not the natural metallic component of carboxylase since magnesium seems definitely to have been established for yeast carboxylase (7). This question of the influence of the type of metallic ion upon soybean carboxylase has been extensively investigated (8), wherein magnesium was shown to be the most active ion of a series examined, with manganese a close second. Accordingly, in the present work on the dissociation and reconstruction of soy bean carboxylase, manganese was employed, but the results obtained are most probably valid, with the exception that the exact stoichiometric porportions cannot be assumed to hold for different ions.

It appears that early in sprouting the combined thiamine rises after 6 hr. and then shows a progressive fall, and this rise and fall is closely paralleled by the acetoin produced and the carboxylase activity shown after added pyruvic acid (Fig. 1). As was previously known (2), the sum of the free and combined thiamine remains constant over the 4 day period. The CO_2 respiration exhibits a gradual rise over the period of sprouting after a sharp initial fall.

Thus, early in growth the metabolism of the seed must require the phosphorylation of thiamine, but this process is completely reversed in about 4 days. Enough is known concerning acetoin to postulate that its production is an index of carbohydrate utilization in the seed. There seems no doubt but that during the 4 day period the seed passes through at least two separate phases of metabolic growth.

SUMMARY

1. The thiamine content of soybean sprouts has been investigated in its several forms.

2. Carboxylase was shown to be present; its activity rises to a maximum shortly after sprouting begins and then falls off.

3. Variations in carboxylase activity are paralleled by variations in the amount of thiamine in the combined form.

4. The general properties of soybean carboxylase have been examined.

5. Manganese was shown to be an effective activator of the enzyme.

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Water-Soluble Methylol Derivatives of Riboflavin [1]

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Riboflavin is very sparingly soluble in water, its solubility near the neutral point being about 0.1 mg. in 1 ml. Alkaline solutions are very unstable. More concentrated solutions have been preparad by dissolving riboflavin together with other compounds capable of increasing its solubility. For this purpose, N-methylacetamide, sodium dehydrocholate, urea, nicotinamide, aliphatic amidines, veratryl alcohol, salts of boric, mono- and dihydroxybenzoic, gallic, aminobenzoic, adenylic, and other acids have been used (1–11).

Water-soluble derivatives of riboflavin include esters with phosphoric, gallic, aminoacetic, phthalic, succinic and levulinic acids, and others (12–16).

A new class of water-soluble riboflavin derivatives is obtained when riboflavin is allowed to react with formaldehyde in a weakly alkaline medium. When riboflavin is suspended in an excess of formaldehyde solution and a small amount of alkali, which by itself is insufficient for dissolving the riboflavin, is added, then all the riboflavin goes into solution in the course of 3 to 4 days at room temperature. Removal of the excess formaldehyde yields a product which is completely soluble in water. The reaction proceeds only in alkaline medium at pH 9 or higher, and any alkali, such as sodium or potassium hydroxide, carbonate or organic base, can be used. For convenience in obtaining the new products in a high state of purity, K_2CO_3 has usually been employed.

The new compounds thus obtained are almost odorless, yellow or orange powders, which dissolve readily in water, giving yellow solutions showing the typical green fluorescence of riboflavin in neutral or weakly acid solution. The fluorescence disappears in strongly acid or alkaline medium, as in the case of pure riboflavin.

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[1] Presented before the Division of Medicinal Chemistry, American Chemical Society, Washington, D. C., August 31, 1948.

The compounds are very soluble in glycerol, especially on heating, less soluble in propylene glycol, very sparingly soluble in methanol and ethanol and insoluble in acetone, chloroform, ether, and hydrocarbon solvents.

Aqueous solutions do not give the reactions of free formaldehyde. Weak acetic acid does not decompose the solutions on boiling, nor does 0.1 *N* HCl at 37°C. However, boiling with strong HCl or H_2SO_4 splits off formaldehyde. A quantitative determination of the formaldehyde thus liberated shows that recovery is never complete, and we have found only up to 50% of the formaldehyde combined originally.[2]

TABLE I

Stability of Methylol Riboflavin Solutions on Repeated Autoclaving

Original assay 4 mg./ml. (fluorometric assay)

pH	Autoclaved			
	1×	2×	3×	4×
3.6	4.2	4.0	—	3.8
4.6	4.0	4.3	3.3	3.2
5.6	3.8	3.3	3.2	2.1
6.9	1.8	1.1	0.3	—
8.1	3.2	2.8	2.0	1.1

$KMnO_4$ does not readily attack the new compounds at room temperature, but oxidizes them rapidly at 50°C. From the oxidation product, a small amount of riboflavin can be recovered.

The stability of the aqueous solutions to heat is dependent on the pH. Table I shows the resistance to repeated autoclaving, as a function of the pH.

The relative percentage of riboflavin and formaldehyde of the reaction products varies with the reaction time, the excess formaldehyde, the quantity of alkali, and the reaction temperature. One can prepare compounds which contain a chemical equivalent of riboflavin as high as 90% and as low as 40% by merely changing these conditions.

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[2] Similar difficulties in the quantitative determination of bound formaldehyde have been reported by Fraenkel-Conrat, Brandon and Olcott (17) for gramicidin, and by Nitschmann and Lauener (18) for casein.

During the reaction of riboflavin with excess formaldehyde, a mixture of compounds, rather than a single substance, is usually formed. We have been able to isolate, by fractional crystallization, two of these compounds in a relatively pure state. One of these contains one mole of formaldehyde combined with one mole of riboflavin, the other contains two moles of formaldehyde per mole of riboflavin.

TABLE II

Solubility and Melting Points of Methylol Riboflavin Compounds

Compound	Solubility *mg./ml.*	Melting point *°C.*
Riboflavin	0.1	275
Monomethylol riboflavin	12	232–234
Dimethylol riboflavin	80	207–209
Trimethylol riboflavin (mixture)	120	135–150

The solubility of the compounds in water depends on the number of formaldehyde groups bound in the molecule. While the lower members show a limited solubility, the higher ones have a strong tendency to

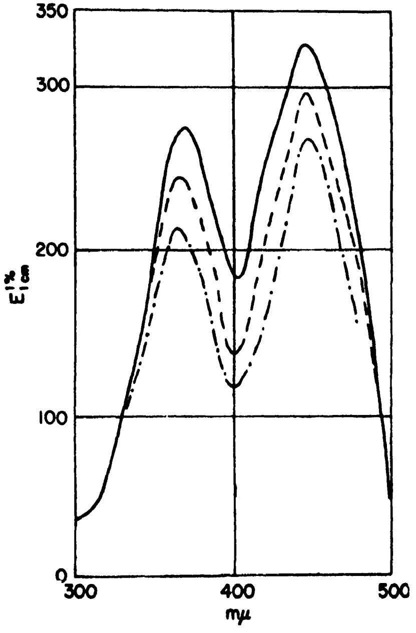

FIG. 1. Absorption spectra. Riboflavin ———; Monomethylol riboflavin — — —; Dimethylol riboflavin —·—·—·

form stable supersaturated solutions. Compounds with 4 or more methylol groups are hygroscopic.

The absorption spectra of riboflavin and of the mono- and dimethylol compounds are shown in Fig. 1. The absorption spectra of the compounds are almost identical with that of riboflavin, except that the maximum at 375 mμ is slightly shifted to shorter wavelengths. The extinction coefficients of the mono- and dimethylol derivatives at the maximum of 445 mμ correspond quantitatively to their respective riboflavin content.

Whereas riboflavin is levorotatory in alkaline and neutral solution and optically inactive in acid solution, the new compounds are all strongly dextrorotatory, both in alkaline and acid medium. The rotation of riboflavin is reversed from levo- to dextro-rotation in borate solution. The rotation of our new compounds increases in borate solution but is not reversed. It is interesting to note that the rotation is highest in the monomethylol compound and decreases with increasing formaldehyde content.

TABLE III

Optical Rotation of Riboflavin and Methylol Compounds

Compound	$[\alpha]_D$ in water	$[\alpha]_D$ in $\frac{1}{2}$ saturated Na borate solution (19,20)
Riboflavin	−114°[a]	+350°
Monomethylol riboflavin	+220°	+325°
Dimethylol riboflavin	+124°	+256°
Trimethylol riboflavin (mixture)	+ 40°	+207°

[a] In *N*/75 NaOH.

Illumination of the methylol compounds in alkaline medium causes decomposition, exactly as in the case of riboflavin. From the illuminated solutions, lumiflavin has been isolated in a pure state, identified by melting point and mixed melting point with lumiflavin from riboflavin.

Acetylation of the methylol compounds with acetyl chloride or acetic anhydride in pyridine gives tetraacetates, thus proving the presence of 4 free hydroxyl groups, the same number as in riboflavin. The acetates are soluble in methanol, ethanol, chloroform, and acetone, and insoluble in water and hydrocarbon solvents. Determination of the optical rotation of the acetates gave equivocal results. In the concentrations which could be used for polarimetric reading, a rotation of a very low order or none at all was observed.

DISCUSSION

The condensation of formaldehyde with riboflavin takes place at the hydroxylated side chain of the latter and not in the ring system. This follows from the qualitative and quantitative study of the absorption spectra and fluorescence, the reversal of the optical rotation, and the formation of lumiflavin.

Additional evidence for our assumption lies in the results of the periodate oxidation (Table IV).

TABLE IV

Periodate Oxidation of Riboflavin and Methylol Derivatives

Substance	Moles HIO_4 used		Moles HCHO isolated	Moles HCOOH isolated
Oxidation time:	1 hr.	4 hrs.	2 hrs.	2 hrs.
Riboflavin	3.9	4.2	1.01 1.07 1.05	1.82 1.77 1.88
Monomethylol riboflavin	3.9	4.2	1.9 1.9	1.41 1.58 1.58
Dimethylol riboflavin	5.1	5.2	1.30 1.27 1.33	2.36 2.48 2.41

Riboflavin and the monomethylol derivative consume 4 moles of periodate. Riboflavin gives 1 mole of formaldehyde[3] and the monomethylol compound 1.9 mole. This indicates that during oxidation the methylol group is split off without being oxidized. The determination of formic acid gives 1.8 and 1.6 mole, respectively, for riboflavin and the monomethylol derivative, which values are close to the theoretical of 2 moles for both compounds. The total yield of 3 moles of oxidation products for riboflavin and of 4 moles for the monomethylol derivative point to the correctness of our formulation for the latter.

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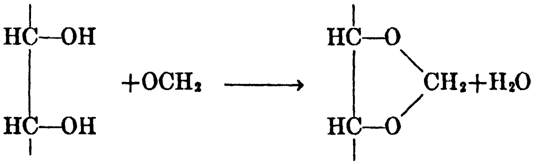
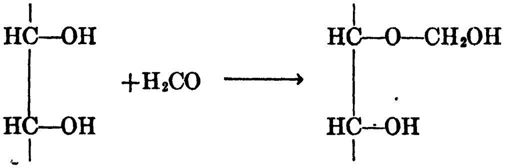
[3] von Euler, Karrer and Becker (21) found only 0.6 mole of formaldehyde, using different experimental conditions.

On the other hand, the dimethylol derivative requires five moles of periodate. This latter value is doubtlessly due to a secondary oxidation of the methylol groups. The quantitative determination gives only 1.3 moles of formaldehyde and 2.4 moles of formic acid, and these values, although in agreement with the assumed structure, are not very close to the expected 5 moles of oxidation products.

There are, in principle, 2 different ways in which formaldehyde can combine with polyhydroxy compounds like riboflavin. The first is condensation of formaldehyde with 2 hydroxyl groups in the 1,2 or 1,3 positions, with elimination of water and formation of a formal (A).



(A)

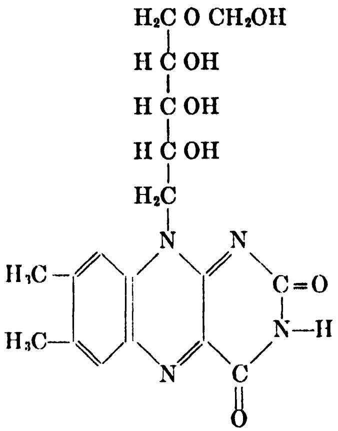


(B)

A second way is the condensation with one hydroxyl group without loss of water (B) and formation of a hemiacetal or hydroxylated ether.

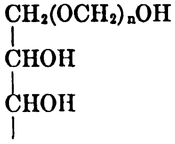
Our compounds cannot have the structure A, because these formals are formed only in acid medium. A formal has also 2 of its original hydroxyl groups blocked, whereas our compounds still have the 4 free hydroxyl groups as in riboflavin. Periodate oxidation of such a formal also would not attack the formal group. Moreover, all formals are less soluble in water than the parent substance, the contraty being the case with our new compounds.

For the foregoing reasons we assign structure B to our new products, which is in good agreement with all their physical and chemical properties. The exact point of attachment of the oxymethylene group or groups has not been determined yet, but it is reasonable to assume that the first mole of formaldehyde condenses with the primary hydroxyl group.



Monomethylol riboflavin

In the case of the di- and trimethylol compounds, a large number of isomers is possible, as the condensation can occur not only on the 4 different hydroxyl groups, but also a multiple chain condensation as in (C) is possible.



$$\begin{array}{l} \mathrm{CH_2(OCH_2)_nOH} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{CHOH} \\ | \end{array}$$

(C)

Biological Activity

As mentioned above, one can prepare condensation products in which the riboflavin content varies from 40% to over 90% when determined by chemical methods. The biological activity of the compounds

TABLE V

Influence of Reaction Time upon the Biological Activity of Methylol Riboflavin

	Assay	
Reaction time *hr.*	Fluorometric *per cent*	Microbiological *per cent*
4	94	55.5
7	92	52.0
8	92	48.8
17	92	34.0
48	88	19
72	66	0.7
96	61	7

is related to this riboflavin content or, in other words, to the reaction time, as seen in Table V.

Preparations with as high as 55% microbiological activity can be obtained upon short reaction time, when only 1 mole of formaldehyde combines with 1 mole of riboflavin. Upon addition of two or more moles of formaldehyde, the activity falls off rapidly.

The new compounds have been tested for their biological activity and toxicity (22) and they have been used in the preparation of synthetic milk diets for nutritional studies on baby pigs (26).[4] They are useful for the preparation of more concentrated solutions than hitherto available, for nutritional or therapeutic applications.

EXPERIMENTAL

Monomethylol Riboflavin

One hundred g. riboflavin and 4 g. K_2CO_3 were suspended in 500 ml. aqueous formaldehyde solution, U. S. P., and stirred at 30°C. for 8 hr. After this time, 5 ml. glacial acetic acid and 1 l. methanol were added with stirring. The solution was freed from undissolved material by filtration, and poured slowly, with vigorous stirring, into 8 l. acetone. The resulting precipitate was filtered off, washed repeatedly with acetone and ether, and dried *in vacuo* at room temperature.

The dry powder was dissolved in hot water to give a solution of 20% (w/v). This was kept in the dark at room temperature for 2 months, after which time a large amount of material had crystallized out. It was filtered off and recrystallized from hot water. A small amount of dark red, insoluble material was filtered off from the hot solution. Recrystallization was repeated 4 times. The monomethylol riboflavin, crystallized in small, orange clusters, m.p. 232–234°C. (dec.), dark above 225°C. The compound retains water of crystallization very tenaciously.

$[\alpha]_D^{23} = +220°$ (in water, c = 0.4),
$[\alpha]_D^{23} = +325°$ (in ½ sat'd sodium borate solution, c = 0.2).

Calcd. for $C_{18}H_{22}N_4O_7 \cdot 3H_2O$ (Mol. wt. 460.43); C, 46.95%; H, 6.27%; N, 12.17%. Found (Dried at 56° @ 0.1 mm., over P_2O_5): C, 47.34, 47.36; H, 6.02, 5.88; N, 12.41, 12.12.[5] Dried at 111°C. @ 0.1 mm.: H_2O: Calcd., 11.7%; found, 7.02%.

Acetate. Five g. monomethylol riboflavin were dissolved in 40 ml. pyridine and 40 ml. acetic anhydride by heating for a short time on a steam bath, then kept over night at room temperature in the dark. The solution was concentrated to 15 ml. and poured into 500 ml. anhydrous ether with stirring. After keeping the mixture in ice water for 2 hr., the precipitate was centrifuged off, washed repeatedly with ether, and crystallized twice from ethanol. The ethanol solutions tend to form a gel upon standing.

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[4] Personal communication from Prof. H. H. Mitchell, University of Illinois, to whom we are indebted for this information.

[5] Microanalyses by Oakwald Laboratories, Alexandria, Va.

Yellow to orange microcrystals, m.p. 192–194°C., sinters at 189°C. Very soluble in acetone, chloroform, and methanol, insoluble in water, ether, and benzene.

$$[\alpha]_D = \pm 8° \text{ (in methanol, c = 1.27).}$$

Calcd. for $C_{26}H_{30}N_4O_{11}$ (Mol. wt. 474.5): C, 54.35%; H, 5.26%; N, 9.75%. Found: C, 54.37, 45.18; H, 5.38, 5.01; N, 9.06, 8.96.

Dimethylol Riboflavin

One hundred g. riboflavin were suspended in 500 ml. formaldehyde solution, U.S.P., 4 g. K_2CO_3 were added, and the suspension was shaken mechanically for 16 hr. at room temperature. At the end of this time the pH of the solution was adjusted to 5.4 with glacial acetic acid, 1 l. methanol was added with stirring, the solution was filtered and run into 8 l. acetone with vigorous stirring. The precipitate was filtered off, washed with acetone and ether, and dried *in vacuo*.

Two hundred forty g. of material obtained in this way from 3 batches of 100 g. each, were dissolved in 2 l. water by heating on the steam bath, and the solution was kept 10 days at room temperature in the dark. After this time, a small dark green precipitate was filtered off, and the solution run into 12 l. acetone with stirring. The resulting precipitate was washed with acetone and ether, and dried. It was then dissolved in glycerol by heating on the steam bath, the glycerol solution filtered through a hot water funnel and kept 3 days at room temperature in the dark, during which time the methylol compound crystallized out. The crystals were filtered off, and recrystallized in the same way from glycerol. After filtering, the solvent was removed by thorough washing with absolute ethanol. An orange, crystalline powder was obtained, m.p. 207–209°C., (dec.), dark at 198°C. The compound retains water of crystallization tenaciously.

$$[\alpha]_D^{18} = +\ 123.6° \text{ (c = 2\% in } H_2O\text{),}$$
$$[\alpha]_D^{19} = +\ 256° \text{ (c = 1\% in } \tfrac{1}{2} \text{ sat'd sodium borate solution).}$$

Calcd. for $C_{19}H_{24}N_4O_8 \cdot 3H_2O$ (Mol. wt. 490.46): C, 46.53; H, 6.17; N, 11.42. Found: C, 46.50, 46.17; H, 6.03, 6.18; N, 10.87, 11.18.

Acetate

The acetate of dimethylol riboflavin was prepared in the same fashion as that of the monomethylol derivative. Orange powder, m.p. 194°C., sinters at 160°C. Optically inactive. Calcd. for $C_{27}H_{32}N_4O_{12}$ (Mol. wt. 604.05): C, 53.63; H, 5.30; N, 9.28. Found: (Rast) 586; C, 53.10, 53.32; H, 5.84, 5.60; N, 9.11, 9.31.

Lumiflavin

Lumiflavin was prepared according to Kuhn *et al.* (23) from riboflavin and from dimethylol riboflavin. Melting point of lumiflavin from riboflavin 338°C. [Kuhn *et al.* (23) give 328°C.; Ellinger and Koschara (24) 315–17°C.] Melting point of lumiflavin from dimethylol compound 336°C. Mixed melting point 337°C.

Oxidation with Periodic Acid

The oxidations were made according to a modification of the method of Reeves (25). All determinations were made at room temperature and in the absence of light. The results are summarized in Table IV.

Formaldehyde

A 75 mg. sample was dissolved in 25 ml. water and 6 ml. *N* $NaHCO_3$ and 6 ml. 0.3 *M* HIO_4 were added. After the desired length of oxidation time, 9 ml. *N* HCl and 50 ml. 0.2 *N* Na arsenite were added, followed by 6 ml. *M* Na acetate and 6 ml. dimedon solution containing 80 mg./ml. in 95% ethanol. The mixture was kept overnight at room temperature, the crystals were filtered, washed with water, dried at 90°C. for 20 min., and weighed.

Formic Acid

One hundred mg. samples were oxidized as described above. After addition of the HCl and arsenite (but before addition of acetate) the solution was made up to 250 ml. volume. From this solution, 50 ml. aliquots were transferred to the apparatus for the determination of formic acid described in Methods of Analysis, A.O.A.C., 6th ed., Washington, D. C., 1945, p. 536, and the determinations carried out exactly as described there.

Periodic Acid Consumed

Accurately weighed samples of 20–30 mg. were placed in 125 ml. Erlenmeyer flasks, 10 ml. water and a drop of dil. NaOH were added in order to dissolve the substance. Then 2 ml. *N* $NaHCO_3$ and exactly 1 ml. 0.5 *M* HIO_4 were added. The mixture was allowed to stand the desired length of time. After this, there were added 1.5 g. $NaHCO_3$ powder, exactly 2 ml. of 0.5 *N* Na arsenite and 1 ml. 20% KI. After standing 10 min., the excess arsenite was back-titrated with 0.1 *N* iodine solution using starch as indicator.

SUMMARY

1. Methylol compounds of riboflavin are formed by condensation with formaldehyde in an alkaline medium.

2. Mono- and dimethylol riboflavin have been obtained in a pure state and their chemical, physical and biological properties are described.

3. The structure of monomethylol riboflavin has been elucidated.

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Book Reviews

Reports of the Biochemical Research Foundation of the Franklin Institute, Volume IX, 1946–47. The Biochemical Research Foundation, *ca.* 250 pp.

This book is a bound collection of reprints. It contains twelve research papers originally published in various scientific journals. One concerns an integrating radiation meter; two are on physical and chemical studies on Southern bean mosaic virus; and nine are on various enzyme-substrate systems, particularly those involving ribonucleic acid and desoxyribonucleic acid. In addition, there are a reprint of the progress report for 1946 by the Director and a review of the literature from 1936 to 1946 on "The Blood Picture of Normal Laboratory Animals," by Mary Virginia Gardner. Notes from the Biochemical Research Foundation for the years 1946 and 1947, originally published in the *Journal of the Franklin Institute*, are also included. A combined subject-author index covering the first nine volumes of "Reports" is appended.

MAX A. LAUFFER, Pittsburgh, Pa.

The Hormones. Vol. I. Edited by GREGORY PINCUS and KENNETH V. THIMANN. Academic Press, Inc., New York, N. Y., 1948. xi + 886 pp. Price $13.50.

This is the first volume of a two volume work which is planned to cover the chemistry, physiology and applications of hormones. It is not a textbook addressed to students. It is a collection of monographs written by experts in their respective fields and, thus, can be classified as an authoritative research reference work. As the editors point out in the preface, their purpose is to bring together into a readily available form information which is scattered through the original literature and earlier review articles.

The first 200 pages of the present volume are devoted to a consideration of topics outside the field of vertebrate endocrinology. Following a brief general historical introduction by the editors, Thimann presents the subject of plant hormones in two chapters. In the first of these the chemistry and physiology of the auxins are discussed. His second chapter is broken down into sections with individual bibliographies dealing with wound hormones, flower-forming hormones, leaf growth substances, plant hormonal properties of vitamins, steroids, and carotinoids, and lastly the less well-defined growth substances in both plants and fungi. Although there may be some question in the minds of many animal physiologists and biochemists as to the desirability of including the so-called phytohormones in a work of this character, since few are also versed in plant physiology, the reviewer believes that most will appreciate this highly condensed but very readable survey of the field.

Non-vertebrate endocrine topics are discussed by Scharrer (hormones in insects) and by Brown (hormones in crustaceans). That this branch of endocrinology has progressed slowly is attested by the fact that only 108 references are made to papers published since the subject of non-vertebrate endocrinology was last reviewed in 1940.

The remaining 600 pages of text are devoted to vertebrate endocrinology. Chapters appear in the following order: Hormones of the Gastrointestinal Tract, by Greengard; The Physiology and Chemistry of the Parathyroid Hormone, by Greep; The Internal Secretion of the Pancreas, by Jensen; Assay of Ovarian Hormones, by Pincus; The Chemistry and Metabolism of the Estrogens, by Pearlman; Chemistry and Metabolism of Progesterone, by Pearlman; Biochemistry of Androgens, by Dorfman; Chemistry and Metabolism of the Adrenal Cortical Hormones, by Heard; Chemistry of Anterior Pituitary Hormones, by Li and Evans; Hormonal Control of Mammary Growth, by Folley and Malpress; and Hormonal Control of Lactation, by Folley and Malpress. These articles are uniformly well done and are documented with extensive citation to the literature. Each chapter has its individual bibliography. The book is equipped with both a subject and author index.

The editors and contributors are to be congratulated on their successful efforts in making this modern work available. In the opinion of this reviewer the book will take its place as a handy desk reference in both advanced teaching and investigation.

J. J. PFIFFNER, Detroit, Michigan

Biology of Pathogenic Fungi. By WALTER J. NICKERSON, Chronica Botanica Company, Waltham, Mass., 216 pp. 46 illus. Price $5.00.

The book was apparently designed to point out the progress already obtained from, and the necessity for continuing, studies on the biochemistry, etiology, and epidemiology of animal diseases of fungal origin. Thirteen workers, under the editorship of Walter J. Nickerson, have contributed individual chapters on specific phases of mycoses. The first portion of the book deals primarily with the etiology and clinical aspects of only four diseases. The chapters are well-written, concise summaries, intelligible and interesting to the nonmedical reader, and since lengthy discussions of taxonomic and nomenclatorial difficulties are omitted, the mycological aspects can be appreciated by other than mycologists. Unfortunately, there is no chapter in which the general principles and present problems in medical mycology are reviewed with historical perspective, although one chapter on geographic distribution is a step in this direction. Integration of past work would help the worker not actively engaged in this field.

The last half of the book develops the thesis that studies on the *in vitro* physiology and biochemistry of fungi may lead to a better understanding of pathogenesis and to practical methods of control. This argument is well developed by the authors through constant suggestion of possible avenues of approach based on existing experimental evidence. Carbon, nitrogen, and mineral requirements are discussed in relation to infection sites and fungus localization in tissues with special emphasis on the mechanism of keratin breakdown by microorganisms. Physicochemical factors affecting growth are similarly treated. Consideration of the metabolic products of pathogenic fungi is brief and deals only with pigments and polysaccharides. An entire chapter is devoted to the lipides of fungi, a class of compounds sometimes overlooked in fungus physiology, and this portion is valuable not only for data on distribution and characteristics of fatty materials but also for the techniques described. The final chapter on respiration and fermentation does not enter into a detailed discussion of enzymatic pathways, but is principally concerned with gaseous exchange, assimilation of carbohydrates, and the possible use of inhibitors of respiration as a control measure. Control measures are more fully discussed in a chapter reviewing the specificity, mode of action, and metabolic effects of sulfonamides and antibiotics on fungi.

The chapters on metabolism are featured by excellent tables and graphs which are utilized to contrast data obtained by several workers. The viewpoint of the entire volume will be refreshing to all students of the fungi.

E. C. STAKMAN and J. M. DALY, St. Paul, Minnesota

Synthetic Methods of Organic Chemistry, Vol. I. 1942–1944. By W. THEILHEIMER, translated from the German by HANS WYNBERG. Interscience Publishers, Inc., New York, N. Y., 1948. (Original German edition published in 1946 by S. Karger, Basel.) x + 254 pp. Price $5.00.

This translation of a recent Swiss book makes available in English the results of a very interesting experiment to classify the synthetic methods of organic chemistry. Selections are taken from the literature according to the usages employed in the years 1942–1944. It is intended to keep the work modernized by issuing other volumes at a later date. Modern techniques in the carbohydrate and steroid fields are well represented by frequent citations. Classification is based upon the type of bond formed, and the author employs a system of symbolization which he has devised. To the reviewer such coinage of new symbols is of doubtful value. One recalls that progress in chemistry was made by the sweeping away by Lavoisier and Berzelius of the supernumerary symbols employed before them. A return to such cryptic mysticism is then more to be deplored than recommended. As a small sop to the backward the author includes a 25-page index of the conventional type. A table of contents would have been of value but has been omitted.

The author has attempted to compress in the short compass of 228 small-sized pages all of the synthetic methods of organic chemistry. Such an ambitious enterprise falls short of satisfactory accomplishment. Nevertheless, it is a step in the right direction and further attempts to modernize and shorten the old "Houben" are to be encouraged. The present American efforts along these lines, as *Organic Syntheses* and *Organic Reactions*, are deficient in that they are non-systematic and incomplete. Let us hope that Dr. Theilheimer and others will continue in these efforts, and that in time a satisfactory solution may be attained.

M. L. WOLFROM, Columbus, Ohio

Insect Microbiology. An Account of the Microbes Associated with Insects and Ticks, with Special Reference to the Biologic Relationships Involved. By EDWARD A. STEINHAUS. Comstock Publishing Company, Ithaca, N. Y., 1946. xiv + 763 pp. Price $7.75.

From the subtitle of this book the reviewer had been led to expect a fundamentally biological approach to, and treatment of, the subject. This hope was strengthened by certain statements in the Introduction; for example: "As will be pointed out in later paragraphs, some of these little-known relationships may ultimately make greater contributions to our knowledge of life than have some of the more dramatic discoveries." (p. 4). "It is now recognized that the academic and 'pure' biology of today pays great dividends years and decades hence. It is not to be doubted, therefore, that present studies on the biologic principles underlying the many associations between microbes and insects will bring forth great scientific profit in the years to come." (p. 6).

Thus prepared for an authoritative and critical account of basic principles, knowledge, and information, upon perusal I found the book a great disappointment.

Perhaps I understand by "biologic relationships" something quite different from what Steinhaus had in mind. To me it implies an evaluation, an attempt at understanding, no matter how primitive or approximate, of the mutual effects of the members of the association upon each other; not merely the enumeration and cataloguing of what microbes have been found on, or in, what insects.

To be sure, the subject matter is extensive; the number of microorganisms that have been found in some sort of association with insects is very large. But the existing information of this kind is mostly incidental, and much of it appears inconsequential. The treatment of the available material by Steinhaus is not such that a clear picture is presented concerning the significance of reported data; the book is chiefly an annotated bibliography, not a critical review or an attempt at synthesis.

The only case of the specific physiological interrelation of microorganisms and insects that has been treated is concerned with the cellulose digestion in termites and roaches, and this treatment is cursory and entirely inadequate; even misleading. It creates the impression that the protozoan fauna occurring in the gut of the insects converts the cellulose, ingested by the host, into glucose, the latter substance being an essential food constituent for the insect. This contention has, several years ago, been exploded by the important studies of R. E. Hungate, whose work is not even mentioned in this context.

Conceivably, the instances of death caused by bacteria in insects might be included among the examples of biological relationships. It is practically the only one that has received much attention in Steinhaus's treatment, but in such a manner as to suggest that, regrettably, numerous bacteria have been isolated from insects without evidence that these microorganisms inflict diseases on the host.

In this connection it should be stated that the method employed for testing the pathogenic potentialities of a microbial culture towards an insect seems very primitive and ill-conceived. Frequently an infection occurs only if the microbes are introduced into the insect body by puncture, and it is doubtful that this manner of inoculation would be common under natural conditions.

There is a virtually complete lack of simple ecological considerations by which it would be possible to relate the occurrence of specific types of microorganisms to the feeding habits of the insects in which they are found. Nevertheless, present knowledge of microbial ecology is, in many instances, quite sufficient to justify the claim that many of the interrelationships, now merely catalogued, could be accounted for on such a basis.

The general deficiencies of the book in connection with the points raised above can be gathered from the passage in which the relations between protozoa and insects are introduced: "The number of species of protozoa known to be associated with insects and ticks is surprisingly large. Whereas most of these arthropods harbor varying numbers of bacteria readily cultivable on artificial media, a microscopic search usually has to be made to detect the protozoa. Nevertheless, the protozoologists have probably investigated the protozoa associated with insects more intensively than the bacteriologists have investigated the bacteria—at least the protozoa appear to have been named and classified more satisfactorily.

"As with the bacteria, very little detailed study has been made of the biologic relationships existing between the protozoa and their insect hosts. Such studies have, in most cases, been limited to investigations of the life cycle or that part of the life cycle which the protozoan spends in the arthropod host. Except in the case of the protozoa of the termite and wood-eating roach, very little study has been made on the physiologic processes involved." (p. 462.)

If this is the situation with respect to the protozoa found in insects, can one, then, expect much of general biological importance when it comes to other microorganisms? Clearly not. And what emerges is some very long and very tiresome lists of names, in the case of bacteria extending over no less than 150 pages. There is not one instance in which the name is accompanied by even an attempt at a satisfactory description; the almost uniform comment following the name of the bacterium is of the kind: "This organism was isolated by . . . from some part of such-and-such an insect." Perhaps the method of printing the names and comments is the only reason why this list occupies more space than the corresponding enumeration of protozoa found in insects (only about 90 pages).

On the whole, the lists and whatever descriptions accompany the names also display a manifestly uncritical attitude. In the case of the bacteria one does rather frequently encounter the remark that an organism given a certain generic name by the original investigator, "should have been placed in the genus *Bacillus*," because spore-formation had been reported. But apart from such trifling criticism the treatment does not indicate that Steinhaus is familiar with the microorganisms dealt with. For example, the description of the "yeasts" of the genus *Chermomyces*, apparently taken from Sulč (p. 365), contains the astounding statements—without comment—that the organisms have a distinct nucleus, that the cytoplasm contains vacuoles, and that multiplication occurs by transverse fission and this in an organism only 1–2 μ long! Also the occurrence of a "highly refractive nucleus" in the yeast-like 'symbiotes' of some weevils (p. 369) goes unchallenged.

That the author has been aware of the deficiencies connected even with the classified enumeration of microorganisms appears from a statement in the Preface: "In places, lists of microbes are given without much associated individual discussion. This has been done for two reasons: first, considering the dearth of information, a discussion of each microbe is impossible; second, thinking such a listing desirable, the author tried to give at least the names of the microbes concerned. For example, hundreds of entomophilic protozoa have been discovered and named, but practically nothing is known of the nature of the biologic relationship between them and their hosts. A similar situation prevails with most of the entomogenous fungi. In the belief that the reader would like to have the names of these microorganisms, the writer has presented them at the risk of appearing to present parts of the book as merely annotated lists." (p. viii). The inference with respect to the reader's interest in names connotes an attitude which the reviewer does not share.

The chapter on "Immunity in Insects" is introduced with the promising remark: "It is perhaps well to call the reader's attention to the fact that a study of the principles of immunity in invertebrates, particularly insects, is not without its applications to the study of vertebrate immunity. This will be pointed out in later paragraphs of the chapter." (p. 555). But then one finds, in the very next paragraph, the statement: "The study of immunity in insects has had only a laggard start. Here lies an almost virgin field waiting to be investigated." (p. 555).

This, again, reveals what can be expected. It is much the same with the rest of the book; there are promises of information of a fundamental nature, but one is always led back to the realization that such contributions still belong to the future.

The above criticism raises the question: why publish so extensive a book on a subject at a time when the cogent information is so far from sufficient for a truly monographic treatment? If the answer were that publication might be an incentive to more concerted and better planned work in the field, the reviewer feels strongly that a much less ponderous and ambitious effort would have been vastly preferable. However, the author expresses the hope "that this volume shall be of use, not only as a reference book to workers in biologic research, but also as a textbook for the classroom." (p. ix). I find it hard to believe that a specialized course in insect microbiology based on the present treatise could be inspiring, and rather fear that it might discourage the better students instead of inducing them to enter the field.

The reasons for my dislike of the use of "biologic," "physiologic," *etc.*, and of "data" as a singular noun can be found in Fowler's "Modern English Usage."

C. B. VAN NIEL, Pacific Grove, Calif.

Crystalline Enzymes. By JOHN H. NORTHROP, MOSES KUNITZ AND ROGER M. HERRIOTT, Member, Associate Member and Associate of the Rockefeller Institute for Medical Research, Princeton, N. J. 2nd ed., revised and enlarged. Columbia University Press, New York, 1948. xxi + 352 pp. Price $7.50.

When "Crystalline Enzymes" appeared in its first edition in 1939, the feeling that it roused in laboratories all over the world was one of deep admiration for this magnificent piece of preparative work. Now, some 10 years later, the second edition, revised and enlarged, will be read with even greater enthusiasm in view of the basic importance of the work with which it is concerned for the development of enzyme chemistry during the past years. It is the reveiwer's opinion that few laboratories have ever contributed so much to our understanding of the nature of enzymes and their action as the Princeton laboratory, and that the book in no small way is a monument of victory for the American school of thought which has maintained against the Willstätter school through many years the idea that enzyme activity is intimately or even inextricably associated with protein structure.

As in the first edition, the volume begins with a chapter called "General Chemistry of the Enzymes," a very elementary treatment that, in the second edition, ends with a valuable list of the enzymes hitherto isolated in crystalline form (Table 3) and with a table giving the amino acid composition of 6 enzymes all prepared in the Princeton laboratory and analyzed by Brand (Table 4). One observes in Table 3 the increasing activity of other laboratories during the past 10 years and finds oneself wishing that more space could have been reserved for a discussion of this work which, in part, is directly inspired by the Princeton school. Table 4, important as it may be for the characterization of these enzymes as proteins, reveals little about the factors determining their catalytic power.

Chapters II, III, and IV deal with pepsin, the enzyme first crystallized by Northrop with pepsinogen and the pepsin inhibitor isolated by Herriott. Here the reader makes his first aquaintance with the solubility test for purity, so extensively used by the Princeton school in the enzyme characterization. The theoretical treatment of this test has been moved to the appendix in the second edition and, while not reaching perfection, it has been much improved. Accordingly, the conclusions drawn from the results of the test have been more or less consistently modified in the rest of the text; greater caution has been exhibited in the postulation of purity than before, and additional evidence by other methods has been collected. To the reviewer's mind the solubility test, when used with care and correctly interpreted, deserves a place among other more recent methods (sedimentation, electrophoresis) for the characterization of proteins. Among new and important accomplishments reported in these chapters is the detailed study of the pepsinogen activation and the isolation of the pepsin inhibitor. (Though the fact is not very important it should be pointed out that the figures in Table 19, section B are quite inexplicable.)

In Chapters V, VI, and VII the authors turn to the proteases of pancreas, namely chymotrypsin, trypsin, carboxypolypeptidase and their precursors and inhibitors. As in the above chapters, there is here an abundance of new experimental material not included in the text of the first edition. A detailed investigation of the homogeneity of chymotrypsinogen is new and the physical and chemical characterization has been much broadened (Table 22). The activation process has been more thoroughly studied (Butler) both in the case of chymotrypsin and trypsin. These cases of protein transformation are of the greatest interest. In how far they represent natural processes cannot as yet be decided. Recent evidence seems to indicate that α-chymotrypsin, for example, is an artifact in the sense that it is unlikely to exist in measurable quantities in the organisms whose intestinal digestion it is supposed to catalyze.

Chapters VIII and IX deal with two more enzymes isolated and crystallized by Kunitz, *viz.*, ribonuclease and hexokinase. The authors have hereby enlarged their field without introducing essentially new methods of isolation. To other workers who have attempted similar isolations there is something almost magic in the way crystalline proteins appear at all stages of their preparations (Chapter IX).

In Chapters X–XII topics are taken up that definitely fall outside of the scope of a book called crystalline enzymes. Bacteriophage was included in the first edition but the isolation of crystalline diphtheria antitoxin (X) and the investigation of the reaction of enzymes with mustard gas (XII) are new. In view of the obvious importance of these investigations, the question of a title is, of course, a minor point.

About the final Chapter XIII in which the senior author presents a working hypothesis for the production of proteins in the cell, the reviewer has very little to say. The problem is hardly ripe for a general discussion of this kind and, although the presentation must be praised for its soberness and intelligence, too many deeply essential points are left untouched. In addition, the quotations are a little erratic—a fault that is found in many places in the book as a whole—and this gives an unexpected distribution of light and shadow over the literature. However the idea of a proteinogen of the type assumed by Northrop to play a central rôle as a precursor for cell proteins may be of help in future investigations of this matter, which is really a matter of life and death.

Finally, attention should be called to the excellent appendix that the second edition has taken over from the first. To the laboratory worker this part of the book is not the least important. Praise should also be given for the many beautiful photomicrographs and, in general, for the setting and arrangement of the book. It is easy to read, which is fortunate in a book that is going to be read as much as this.

K. Linderstrøm-Lang, Copenhagen, Denmark

Calculations of Quantitative Analysis. By Philip W. West, Head, Division of Analytical Chemistry, Louisana State University, Baton Rouge, La. The Macmillan Co., New York, 1948. viii + 162 pp. Price $2.75.

This book consists of 8 chapters, an appendix, and an index. In the introductory chapter, the author gives an excellent review of certain portions of inorganic chemistry, especially that part dealing with valence and equivalent weights, both of which are so important in quantitative analysis and so frequently forgotten or not properly understood by the student from earlier chemistry courses. Dr. West takes great pains in correlating concepts learned in inorganic chemistry to quantitative analysis. Thus, on p. 9, the author shows the application of the weight laws to the development of gravimetric factors.

Following the introductory chapter, chemical factors and the fundamental calculations of gravimetric analysis are discussed. The author has a convenient method, at the end of each chapter, of dividing work problems as well as typical laboratory calculations into general types.

Ch. III deals with fundamental calculations of titrimetric analysis and solution strength, exemplifying both direct and back-titration methods. It is true that the method of expressing concentration of a solution by its titer is probably the simplest and most direct, but the designation in terms of normality is the one used most extensively in volumetric analysis, and is the basis for most calculations in this important subject. This reviewer feels that too much space is devoted to the titer rather than the normality methods of calculation.

Ch. IV involves calculations of percentages, specific gravity-percentages, molar, normal, and titer solutions, while Ch. V deals with oxidation-reduction problems.

Ch. VI is entitled "Miscellaneous Calculations" and treats of adjusting strengths of solutions, concentrations of solution mixtures, double indicator titrations, factor weights in gravimetric analysis, factor weights in titrimetric analysis, indirect analysis, relationship between solutions used in double decompositions, and oxidation-reduction reactions and the calculation of molecular formulas. In this chapter, the author has omitted the wet-dry basis and titration curve calculations.

Ch. VII deals with chemical equilibria and the law of mass action, including ion concentrations of weak electrolytes, hydrogen ion concentration and pH, common ion effect, solubility products, polybasic acids, and hydrolysis of salts and buffers.

The last chapter is devoted to the reliability of analytical results, which includes computation rules, precision and accuracy, errors, statistical methods, and rejection of a result. This chapter is handled well. As a complete discussion of errors is impossible in such a text, the author picked a few of the most essential topics and supplemented these with about 5 references.

As specified in the preface, the student's interest and imagination is stimulated by including a number of problems bearing directly on industrial applications from such fields as metallurgy, geology, and water analysis. In these problems sufficient information is given to enable the student to understand the principles of the determination involved as well as the nature of the procedures.

The appendix consists of tables of ionization constants for weak acids and weak bases, solubility products, molecular weights of some of the common compounds; international atomic weights, and a graphic log table. In this reviewer's opinion, several important tables have been omitted, *e.g.*, those of densities, solubilities of bases and salts in water, redox potentials, and a comparison of metric and English units of measurement. In addition, although methods for determining gravimetric factors are given in Ch. II, the reviewer believes that a table of the common gravimetric factors and their logs should be included.

LEO K. YANOWSKI, New York, N. Y.

Erratum

Volume 18, Number 3, p. 494.

Reference 8 should be van Wagtendonk, Schocken and Wulzen, *Arch. Biochem.* **3**, 305 (1944).

New Specific Color Reactions of Hexoses and Spectrophotometric Micromethods for their Determination [1]

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INTRODUCTION

The only color reaction which hitherto can be considered as a sufficiently sensitive and specific test for aldohexoses and ketohexoses (1) is that with diphenylamine and HCl. However, certain other carbohydrates and their derivatives interfere with the reaction. The carbazole reaction recommended by Gurin and Hood (2) and Seibert (6) for the identification of hexoses in mixtures gives fairly satisfactory results when applied to purified polysaccharides or plasma proteins, but lipides, sulfhydryl compounds, and hexuronic acids interfere with it (3).

In 1946 a new color reaction of sugars with SH compounds and H_2SO_4 was described (4), which can be used for the detection and determination of hexoses in the presence of an excess of other sugars and tissue constituents.

In this report two modifications of the cysteine reaction of hexoses are described, which differ from each other in the concentration of the acid, the time of heating and addition of cysteine. These two proce
yield three reactions, the reaction products of which di
ably in their absorption spectra. All three reactions
quantitative determination of hexoses. Their c
to determine 3 hexoses in mixture with each
other classes of sugars. We designate in th
dures as CyRI and CyRII.

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[1] This work was supported by a grant of t
Division.

EXPERIMENTAL

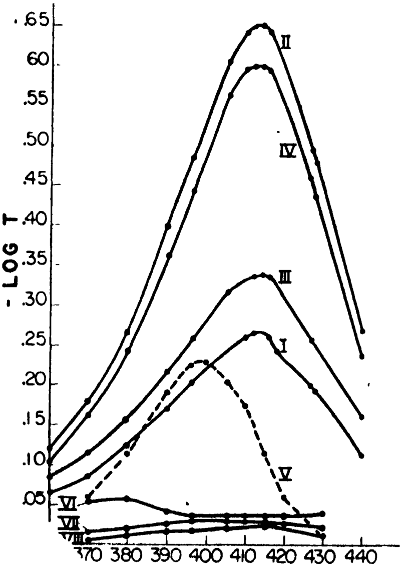
A. CYRI

a. Procedure

4.5 cc. of a mixture of 1 part of H_2O and 6 parts of H_2SO_4 (Baker and Adamson C.P.) are pipetted into a 16 × 150 mm. test tube immersed in ice water. After a few minutes, 1 cc. of the solution to be tested is added with shaking and continuous cooling in ice water. After a few minutes the tube containing the reaction mixture is placed in tap water for a few minutes and then for exactly 3 min. in a boiling water bath, and cooled afterwards in tap water. One-tenth cc. of a 3% solution of cysteine hydrochloride is then added and the mixture shaken.

b. The Primary Reaction in CyRI as a general Reaction of Hexoses

1. Absorption Spectra. A few minutes after addition of cysteine a yellow color appears. The four hexoses investigated (glucose, galactose, mannose, and fructose) show considerable absorption between 370 and 440 mμ--with a maximum at 415 mμ. The absorption curves are almost symmetrical with respect to the maximum and differ only in height for the 4 hexoses (Fig. 1). In the course of time the absorption at



various sugars in PCyRI.

I	Fucose	1 mg.-%	V
II	Thymonucleic acid	50 mg.-%	VI
'I	Yeast adenylic acid	10 mg.-%	VII
	Glucuronic acid	10 mg.-%	VIII

415 mμ decreases slowly and, after 48 hr., at room temperature, the maximum disappears. The reaction responsible for this characteristic absorption will be designated by PCyRI.[2]

2. Specificity of the Reaction. The reaction is not entirely specific for hexoses, since methylpentoses also show a yellow color (5). The absorption curve for the latter, however, is quite different, with a maximum at 400 mμ, and the product is stable. Pentoses give a red color, with a maximum around 540 mμ; the absorption curve is almost horizontal between 380 and 430 mμ. The products from hexuronic acids and desoxyribose likewise show little change in absorption between 380 and 430 mμ.

c. The Secondary Reactions in CyRI as Reactions for Individual Sugars

The primary reaction products of CyRI are unstable and the absorption maximum at 415 mμ decreases slowly and disappears almost completely in the course of 48 hr. at room temperature. At the same time, other color reactions appear, which are different for the different hexoses. Galactose and sorbose give blue colors, glucose and fructose green, and mannose gives a yellow color. Apparently two products with different absorption spectra are involved. One of the products has an absorption maximum at 600 mμ and appears blue, the other product (or probably group of products) shows an intensive absorption between 380 and 420 mμ with no characteristic maximum and appears yellow. The ratio of intensities of absorption of these two compounds is different with different sugars and its variations are responsible for the difference in color. D_{600} (measured with a Beckmann spectrophotometer) for galactose is 7 times that for mannose and 3 times that for glucose, while the absorption in the blue due to the yellow compound is almost equal for all 3 hexoses. The bluish green of glucose and

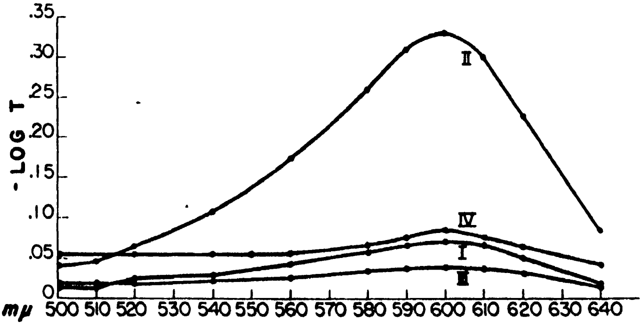


FIG. 2. Absorption spectra of hexoses in SCyRI.

Glucose	5 mg.-%	I	Mannose	10 mg.-%	III
Galactose	10 mg.-%	II	Fructose	5 mg.-%	IV

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[2] The reaction of methylpentoses described before as CyR3 is obtained by a procedure identical with that described here as CyRI. We will use from now on the designation PCyRI for the reaction of methylpentoses as well as that of hexoses and distinguish the two when necessary asPCyRI$_M$ and PCyRI$_H$.

fructose thus appears as a mixture of blue and yellow, while, in the case of mannose, the blue component is barely perceptible (Fig. 2). The secondary reaction of hexoses in CyRI which shows a maximum at 600 mμ will be designated as SCyRI.

B. CYRII

a. Procedure

To 0.9 cc. of the solution in a test tube are added 0.1 cc. of a 3% solution of cysteine hydrochloride and 5 cc. of a mixture of 190 cc. of H_2O and 450 cc. of H_2SO_4 (75 vol. of acid/100 vol. of the mixture). The tube is heated for 10 min. in a boiling water bath and then cooled in tap water and left at room temperature for 48 hr.

b. Absorption Spectra of Various Sugars in CyRII

As in CyRI at least two reaction products appear in CyRII. One develops slowly in the course of 48 hr. at room temperature and has a sharp absorption maximum at 600 mμ. This product is found with all 4 hexoses and appears to be the same as the blue product of SCyRI. The other product is formed more rapidly and may be observed at the end of the heating period. It is practically colorless but shows a considerable absorption in the ultraviolet between 320 and 400 mμ. The intensity of absorption for the reaction mixture in this region increases slowly for 24 hr. at room temperature. The reaction responsible for this absorption will be designated as PCyRII. The absorption curve and the position of the maximum are different for every hexose. Glucose and galactose show a flat maximum between 320 and 330 mμ, levulose shows one at 340 mμ and mannose one at 355 mμ. The latter maximum is very flat and extends almost from 340 to 370 mμ (Fig. 3).

c. Specificity of PCyRII

Sugars other than hexoses also show considerable absorption in the ultraviolet. The absorption of methylpentoses and desoxypentoses is much weaker than that of hexoses and the absorption curves are practically horizontal lines between 350 and 400 mμ, and rise slowly from 350 to 320 mμ. In the case of pentoses and hexuronic acids the absorption increases continuously and steeply from 400 to 320 mμ. This rise probably continues beyond 320 mμ. However, it was not investigated further.

C. DETECTION AND QUANTITATIVE DETERMINATION OF HEXOSES BY CYRI AND CYRII

a. Measurement of Absorption by the Compensation Procedure

The intensive blue color of galactose and sorbose in SCyRI and II enable one to detect these sugars even in the presence of an excess of other sugars. But these tests do not allow the detection of other hexoses in mixtures with each other or galactose. Nor is the yellow color due to PCyRI characteristic for hexoses. In general, the detection of hexoses as a class and of individual hexoses in mixtures can be carried out safely only by quantitative spectrophotometric measurements of the absorption at appropriate wavelengths. Many organic substances present in tissues and body fluids react with H_2SO_4. The products of these reactions often absorb strongly in the blue, violet and ultraviolet. This absorption contributes to the absorption due to the reaction of sugars with cysteine in the unknown solution. To make allowance for this complication it is necessary to run with every sample of the unknown to which cysteine is added (cysteine sample) another sample to which are added only H_2SO_4 and H_2O (water sample) instead of cysteine solution. The absorption of the first sample is measured against a water blank with cysteine and of the second against one without cysteine. In quantitative determinations of sugars, the same compensation procedure is carried out on the standard solutions.

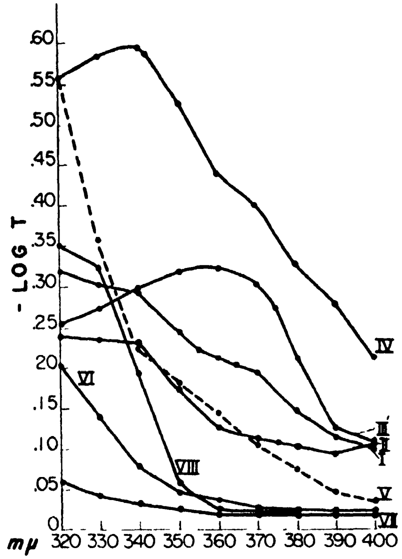

FIG. 3. Absorption spectra for various sugars in PCyRII.

Galactose	10 mg.-%	I	Ribose	10 mg.-%	V
Glucose	10 mg.-%	II	Glucuronic acid	20 mg.-%	VI
Mannose	10 mg.-%	III	Thymonucleic acid	10 mg.-%	VII
Levulose	10 mg.-%	IV	Fucose	5 mg.-%	VIII

b. Spectrophotometric Test for Hexoses in General

1. Detection in Presence of Other Sugars by the Absorption Spectrum of PCyRI. In this reaction all 4 hexoses so far investigated show an identical shape of the absorption curve and position of its maximum (Fig. 1). The absorption curves of pentoses, hexuronic acids and desoxypentoses are practically horizontal lines between 380 and 430 mμ. Their presence in solution will not affect, therefore, the shape and maximum of the curve of hexoses, except that it will cause a slight elevation of the whole curve. Methylpentoses, however, with their sharp maximum of the absorption curve at 400 mμ will cause a distortion of the curve of hexoses. To eliminate this disturbance we determine first the concentration of methylpentoses in the solution according to the procedure described previously (5). As CyRI of methylpentoses is identical with that of CyRI of hexoses, this determination can be done on the same sample which is used for the detection of hexoses. From the known concentration of methylpentose in the unknown and the absorption of a standard solution of methylpentose at a series of wavelengths between 380 and 430 mμ the absorption due to the methylpentose in solution is calculated and deducted from the absorption values of the solution. The corrected absorption plotted against wavelength gives the absorption curve of hexoses, presumably present in solution. This somewhat involved procedure seems to be the only way to detect aldohexoses in very small amounts in solutions containing other sugars.

2. The Absorption Increment Between Two Wavelengths in PCyRI as Test for Hexoses. A less elaborate procedure to provide evidence for the presence of hexoses in solution consists in determining the density of a solution at two wavelengths at which the extinction coefficients of other sugars are equal. Thus D_{380} and D_{415} in PCyRI are equal for methylpentoses and, of course, for the other sugars which show a uniform intensity of absorption between 380 and 430 mμ (Fig. 1 and Table I). D_{415}–D_{380} is, therefore, zero for all classes of sugars except hexoses, for which it is positive. Ten γ of hexoses in 1 ml. can be detected safely by this difference.

3. Detection of Individual Hexoses in Mixtures with Other Sugars. CyRII can be used as a simple spectrophotometric test for mannose and galactose, which enables one to detect 1 part of these sugars in 5 parts of other hexoses and in the presence of an excess of other sugars. The absorption curve for glucose is almost horizontal between 400 and 375 mμ, and then rises slowly at an increasing rate towards 320 mμ. The curve of mannose, on the contrary, rises rapidly between 400 and 375

TABLE I

Densities at 380 and 415 mμ and Their Differences for Solutions of Various Sugars in PCyRI

Exp	No.	Substance	Concentration in mg.-%	$D_{380} \times 1000$	$D_{415} \times 1000$	$D_{415}-D_{380} \times 1000 \times 1000$
I.	a.	Glucose	5	145	370	225
	b.	Glucose	2.5	74	192	118
	c.	Glucose	1.25	40	96	56
	d.	Mannose	5	68	162	94
	e.	Fructose	5	159	400	241
	f.	Yeast adenylic acid	10	22	27	5
	g.	Desoxyribo-nucleic acid	50	56	37	−19
	h.	Glucuronic acid	10	11	26	15
II.	a.	Hyaluronic acid	10	27	33	6
	b.	Chondroitin sulfuric acid	10	20	4	−16
	c.	Heparin	10	69	92	23
III.	a.	Glucose	2.5	84	196	112
	b.	Glycogen	2.5	91	213	122
	c.	Desoxyribo-nucleic acid	20	45	45	0
	d.	Muscle adenylic acid	10	9	3	−6
IV.	a.	Galcatose	5	84	201	117
	b.	Galactose	2.5	48	111	63
	c.	Glucose	2.5	68	177	109
		Glucose	2.5			
	d.	+galactose	2.5	110	279	169
	e.	Fucose	1			
		+Acetylglucosamine	5	121	128	7
	f.	+Galactose	2.5	160	220	60
V.	a.	Rhamnose	1	115	113	−2
	b.	Arabinose	4	18	18	0
	c.	Fucose	0.5			
		+Arabinose	4	78	78	0

TABLE II

Densities at 605 mμ for Different Sugars in Secondary CyRI in Presence of Mannose

Exp.	No.	Substance	Concentration in mg.-%	$D_{605}\times 1000$
I.	a.	Galactose	1	71
	b.	Galactose	2	130
	c.	Galactose	4	273
	d.	Glucose	4	110
	e.	Glucose	8	222
	f.	Glucose	4	
		+Galactose	4	388
	g.	Fucose	1	4
	h.	Acetylglucosamine	5	4
II.	a.	Galactose	4	261
	b.	Galactose	4	
		+Serum albumin	100	262
	c.	Galactose	4	
		+Serum albumin	50	265
III.	a.	Galactose	8	576
	b.	Galactose	4	281
	c.	Galactose	2	155
	d.	Mannose	10	
		+Muscle adenylic acid	40	6
	e.	Fucose	5	5
	f.	+Galactose	4	269
		+Galactose	4	
	g.	+Muscle adenylic acid	40	250
IV.	a.	Galactose	4	325
	b.	Galactose	2	164
	c.	Galactose	1	87

mμ and then flattens out suddenly. The curve for galactose differs from the two others, the average rise along the ordinate being equal in the two ranges, 400–375 and 350–375 mμ (Fig. 3). These geometrical differences in the 3 absorption curves may be represented by the difference of the two density increments, $D_{375}-D_{400}$ ($\Delta D_{375,400}$) and $D_{350}-D_{375}$ ($\Delta D_{350,375}$). $\Delta D_{375,400}-\Delta D_{350,375}$ is zero for galactose, positive for mannose and negative for glucose (Table III). It is also substantially zero for methylpentoses (fucose and rhamnose) and for desoxyribonucleic acid. It is slightly negative for hexuronic acids and pentoses, so that, in the presence of these sugars in an amount not exceeding that of mannose, the test becomes somewhat less sensitive, but by no means inapplicable. Serum albumin in 20-fold excess does not affect the test. Calculation of the ratio: $\frac{D_{600}}{\Delta D_{350,\,375}}$ provides a test for galactose. This ratio is 4.5 for galactose, 1.4 for glucose, 1.1 for mannose, and 1 for fructose. Any value over 1.4 indicates, therefore, the

TABLE III

Density at 610 mμ and Density Increments between 350 and 375 mμ and between 375 and 400 mμ for Various Sugars in CyRII.

Exp.	No.	Substance	Concentration in mg.-%	A $D_{375,400} \times 1000$	B $D_{350,375} \times 1000$	A–B	D_{610}	$\frac{D_{610}}{B}$
I.	a.	Galactose	10	60	56	+4		
	b.	Glucose	10	0	67	−67		
		a+b		74	120	−46		
	c.	Mannose	10	163	39	+124		
		a+c		232	99	+133		
II.	a.	Galacturonic acid	20	13	44	−31		
	b.	Fucose	2	−10	−8	−2		
	c.	Galactose	10	56	54	+2		
	d.	Galactose	10					
		+Serum albumin	100	61	65	−4		
	e.	Desoxyribo-nucleic acid	10	2	5	−3		
III.	a.	Galactose	10	65	56	+9	166	3
	b.	Galactose	5	39	36	3	99	2.76
	c.	Fucose	5	−6	−11	+5	3	0
		b+c		31	14	+17	61	4.3
	d.	Fucose	10	−15	−19	+4		
	e.	Mannose	10	135	8	+127	19	0.15
	f.	Mannose	5	70	8	+62	9	0.15
	g.	Mannose	2.5	33	3	+30	5	0.17
IV.	a.	Galactose	10	70	64	+6	179	2.80
	b.	Yeast adenylic acid	40	77	121	−44	−7	
		a+b		161	165	−4	146	0.88

TABLE IV

Densities at 370 and 400 mμ and Their Difference for Solutions of Varying Concentration of Different Sugars in PCyRII

Exp.	No.	Substance	Concentration in mg.-%	$D_{370} \times 1000$	$D_{400} \times 1000$	$D_{370}-D_{400} \times 1000$
I.	a.	Galactose	10	167	89	78
	b.	Glucose	10	114	98	16
		a+b		290	188	102
	c.	Mannose	10	275	80	195
		a+c		437	157	280
II.	a.	Xylose	10	102	9	93
	b.	Glucose	10	125	121	4
	c.	Glucuronic acid	20	14	11	3
	d.	Levulose	10	377	179	198
III.	a.	Galactose	10	178	90	88
	b.	Galactose +Serum albumin	10 100	179	85	94
IV.	a.	Fucose	5	25	30	−5
	b.	Galactose	4	76	37	39
		a+b		89	57	32
	c.	Rhamnose	5	21	17	−4
	d.	Galactose	8	137	63	74
	e.	Mannose	8	206	50	156
	f.	Mannose	4	107	26	81
	g.	Mannose	2	51	13	38
V.	a.	Galactose	10	148	69	79
	b.	Glucose	10	74	57	17
	c.	Glycogen	9	81	66	15
	d.	Fucose	5	24	11	−13
	e.	Fucose	2.5	13	7	−6
	f.	Rhamnose	10	35	32	−3
	g.	Blood group A	50	218	119	99
VI.	a.	Mannose	5	139	36	103
	b.	Glucose	10	130	106	14
		a+b		250	131	119
	c.	Serum albumin	100	11	8	3
		a+c		133	28	105
	d.	Muscle adenylic acid	40	16	4	12
		a+d		157	35	122
	e.	Galacturonic acid	20	34	17	17

presence of galactose or some other sugar not investigated. The presence of pentoses decreases the sensitivity of the test, other sugars and serum albumin in 20-fold amount do not (Table III).

As the differences of the density increments for mannose and glucose have opposite signs, their sums may become zero and mixtures of the sugars in certain proportions may behave like galactose. The value for: $\frac{D_{600}}{\Delta D_{350,\,375}}$ in such a case will reveal the true situation. Fifty γ/cc. of mannose and 20 γ/cc. of galactose are the minimum amounts which can be detected by these tests.

c. Quantitative Determination of Hexoses in Mixtures with Each Other in Presence of Other Sugars

1. Principle. To determine quantitatively several hexoses present in one solution we need as many independent equations derived from as many measurements of densities of the unknown as there are hexoses in solution. It is not always possible in the presence of other sugars to obtain the absorption due to hexoses only by measurements at single wavelengths. To eliminate the influence of other classes of sugars we have to substitute measurements of densities at a single wavelength by measuring increments of densities between two wavelengths. These are chosen in such a way that the density increment between them is zero for all sugars present in solution except hexoses. To be appropriate measures for the concentration of hexoses these density increments must be proportional to their concentration and must show an additional behavior in mixtures of various hexoses. Furthermore, the corresponding extinction coefficients and increments of extinction coefficients for various hexoses must differ to an adequate extent from each other.

It was shown before that the density increment D_{415}–D_{380} (designated $\Delta D_{415,\,380}$) for hexoses in PCyRI is not influenced by the presence of other sugars. Nor has 0.1% of serum albumin any significant effect. As can be seen from Table I, $\Delta D_{415,\,380}$ is strictly proportional to the concentration of hexoses in concentration between 0.001 and 0.006% and it shows an additive behavior in mixtures of hexoses.

In PCyRII the density increment between 370 and 400 mμ ($\Delta D_{370,\,400}$) is strongly positive for pentoses, zero for desoxyribose and barely significant for methylpentoses and hexuronic acid. It is proportional to the concentration of hexoses and additive in mixtures. It is not affected significantly by 0.1% serum albumin.

As noted before in SCyRI the absorption maximum at 600 mμ seems to be highly characteristic for hexoses. D_{600} however is not proportional to the concentration of the sugar. It increases more than the latter and, with mixtures of several hexoses, it is higher than the sum of D_{600} for the separate components. This anomaly disappears, however, when 0.01% of mannose is added to a solution of a hexose. In this case, the maximum of absorption of SCyRI shifts to 605 mμ. Although mannose alone shows a low D_{605} in mixtures with other hexoses it makes D_{605} proportional to the concentration of hexose and additive above the concentration of 0.002%. Below that range, a very slight deviation from proportionality appears which can be easily corrected (Table II). In the presence of at least 0.01% of mannose, therefore, D_{605} appears to be an appropriate measure of SCyRI. It can be seen from Table II that pentoses, methylpentoses and desoxyribose do not show any significant absorption at 605 mμ. In mixture with galactose, fucose has no significant effect as long as its concentration is less than half of that of the hexose. Two-hundredths per cent of muscle and 0.04% of yeast adenylic acid decrease D_{605} of galactose of 0.004%, 3 and 4%, respectively.

2. Procedures. PCyRI and PCyRII are carried out for quantitative determinations in exactly the same way as described for qualitative purposes. In SCyRI, however, 0.2 cc. of a 0.05% mannose solution is added to 0.8 cc. of the unknown as well as to the standard and the blank. In general, 3 samples of the unknown, of the standard and blank are used for every determination. To two of them 0.1 cc. of cysteine solution is added after heating (duplicates of cysteine samples). The third serves as water sample. The total concentration of hexoses in the unknown should be 10–50 γ/cc. for PCyRI and SCyRI and 50–200 γ/cc. for PCyRII. The readings on the Beckmann spectrophotometer should be done for the first reaction not later than 3 hr. after addition of cysteine. Because of the slow decrease in absorption the unknown should be read simultaneously with the standard. A 0.005% solution of any of the hexoses can serve as standard. In the two other reactions, the readings are carried out 48 hr. or later after the addition of cysteine. For SCyRI a solution of glucose or galactose, for PCyRII a solution of mannose or galactose is used as standard. Test tubes of the same diameter (16 × 150) should be used for all samples. The water bath should be boiling so vigorously that, after immersion of the tubes, the boiling should not stop.

3. Calculation. If X, Y, Z are the 3 hexoses present in solution and a solution of Z is used as standard, the concentrations c_x, c_y, c_z of every hexose can be calculated from the following 3 equations:

$$\text{I. } c_x \frac{\epsilon_x{}^1}{\epsilon_z{}^1} + c_y \frac{\epsilon_y{}^2}{\epsilon_z{}^1} + c_z = D_R{}^1,$$

$$\text{II. } c_x \frac{\epsilon_x{}^2}{\epsilon_z{}^2} + c_y \frac{\epsilon_y{}^2}{\epsilon_z{}^2} + c_z = D_R{}^2,$$

$$\text{III. } c_x \frac{\epsilon_x{}^3}{\epsilon_z{}^3} + c_y \frac{\epsilon_y{}^3}{\epsilon_z{}^3} + c_z = D_R{}^3.$$

$D_R{}^1$, $D_R{}^2$, $D_R{}^3$ are the ratios of the density or density increments of the unknown and the standard in the 3 respective reactions used for the determination, while the symbols: $\epsilon_x{}^n$, $\epsilon_y{}^n$, $\epsilon_z{}^n$ ($n = 1$, 2 and 3) are either the extinction coefficients of the 3 hexoses at 605 mμ in SCyRI or the respective increments of extinction coefficients between 415 and 380 mμ in PCyRI and between 370 and 400 mμ in PCyRII. ϵ of every sugar varies somewhat from one determination to another. In Eqs. I to III, however, the coefficients on the left side are ratios of extinction coefficients which are more constant. The values for these ratios for all 3 reactions are given in Table V.

TABLE V

Ratios of Extinction Coefficients of Various Hexoses to that of Glucose in the Three Reactions

Hexose	Ratio of extinction coefficients in		
	PCyRI	SCyRI	PCyRII
Mannose	0.40	0.23	18.30
Galactose	0.51	2.50	7.70
Fructose	1.12	0.75	18.00

4. Selection of Appropriate Reactions in Special Cases. If the unknown solution contains only two hexoses, the choice of the two reactions most appropriate for the determination depends on the nature of the sugar.

a. Determination of Galactose and Glucose or Mannose in the Presence of Other Classes of Sugars. In this case the reactions PCyRI and SCyRI are chosen. $\Delta D_{415,\,380}$ in the first is about twice as great for glucose as for galactose or mannose and practically zero for all other classes of sugars. The ratio of D_{605} in SCyRI for galactose, glucose and mannose is 2.5; 1; 0.23. The combination of these two procedures permits one, therefore, to determine galactose and one of the other hexoses fairly accurately in solutions containing 10 γ or more/cc. of every hexose (Table V).

b. Determination of Mannose in the Presence of Glucose. In this case the two most appropriate reactions are PCyRI and PCyRII. $\Delta D_{370,400}$ in the latter reaction is 19 times greater for mannose than for glucose. The content of mannose in the solution should be at least 20 γ/cc.

5. Determination of Hexoses in Polysaccharides. As can be seen from Tables I, II, and III, D_{605} in SCyRI, $\Delta D_{415,430}$ in PCyRI and $\Delta D_{370,400}$ in PCyRII for glycogen do not differ significantly from those for glucose. The glycosidic linkages in polysaccharides, therefore, do not change in principle the reactivity of hexoses in those 3 reactions. Thus, polysaccharides can be determined without prior hydrolysis.

6. Determination of Hexoses in Presence of Adenosine-5-Phosphate. Crude tissue extracts contain, in general, considerable amounts of adenosine-5-phosphate in free or combined form. PCyRI is not affected by this compound, but D_{605} in SCyRI of hexoses shows a decrease of a few per cent in presence of an excess of this compound. On the other hand, $\Delta D_{370,400}$ of PCyRII is sligthly positive for adenosine-5-phosphate, although 25 times smaller than that of mannose. For accurate determinations of hexoses in crude tissue extracts, it is, therefore, necessary to correct for this influence of adenosine-5-phosphate. This requires its determination in the extract.

7. Simple Test for Pentoses and Hexuronic Acids in Presence of Hexoses. Pentoses not substituted in position 5, and hexuronic acids when present in high amounts, may interfere with the determination of hexoses by PCyRII. It is, therefore, important to detect their presence in the unknown solution. This can be done easily on the same sample in which hexoses are determined by CyRII by measuring D_{320}–D_{340} of the solution 2 hr. after the end of the heating period and comparing it with D_{320}–D_{340} 48 hr. later. Any increase in this value indicates the presence of significant amounts of pentoses or hexuronic acids.

8. Influence of Chloride Ions on PCyRI and SCyRI. Chloride ions influence the two reactions when present in concentrations comparable to those of living cells and body fluids. PCyRI is strongly increased even by *M*/10 NaCl in solution. *M*/200 NaCl increases $D_{415,380}$ by 20% However, at this concentration of chloride ions the percentage increase is the same at various concentrations of the hexose. This is not the case at concentrations of chloride ions higher than *M*/200. To eliminate this influence of NaCl, it is necessary to add to the standard solution the same amount of NaCl as is present in the unknown or better to use an internal standard.

SCyRI is decreased by chloride ions. The decrease for various concentrations of hexoses is only about 5% for $M/100$ NaCl. In using SCyRI on blood extracts it is, therefore, necessary to make the final dilution of the blood 20-fold against the original blood and use a standard containing $M/175$ NaCl.

9. Influence of Impurities in the H_2SO_4 on PCyRI. Certain products of unknown nature present in H_2SO_4 not highly purified increase considerably the PCyRI of hexoses. It is, therefore, not advisable for example to use H_2SO_4 free of N and As. To obtain comparable results, one and the same brand of H_2SO_4 should be used for all determinations. In experiments here reported H_2SO_4 c.p. of Baker and Adamson was used exclusively.

DISCUSSION

The three reactions of hexoses with cysteine promise to be of considerable value for the investigation of the so-called neutral mucopolysaccharides in animal tissues and body fluids and for polysaccharides of bacteria. They can also be useful for the identification and analysis of complex mixtures of polysaccharides like those in urine and red cells and for the determination of galactose in blood. In all investigations of this type it is always necessary to ascertain by recovery experiments whether the substrate influences the intensity of the reaction. If the latter is the case, the use of an internal standard is indicated. In conjunction with these reactions, the methods described previously for the detection and determination of hexuronic acids and methylpentoses are of use. Pentoses and methylpentoses can also be detected by the CyRII in the same sample that is used for the determination of hexoses. A procedure for this purpose will be reported in a subsequent paper.

Compared with the diphenylamine reaction CyRI and II have the advantage of higher sensitivity and of not being affected in their accuracy by the presence of fructose (or its esters), trioses and pentoses. Furthermore, PCyRI permits the determination of methylpentoses in the same sample as hexoses. On the other hand, the carbazole reaction of Gurin and Hood (2) appears to be less influenced than the cysteine reactions by the presence of high amounts of proteins, and can be used for the determination and identification of hexoses in alcohol-precipitated serum (6). The diphenylamine reaction is valuable for the detection and determination of fructose and its ester.

But apart from this use for particular purposes these older methods can be applied for checking the results of the determinations of hexoses by the cysteine procedures. It was pointed out in a previous report that the validity of identifications of sugars by color reactions is considerably enhanced if quantitative determinations of these sugars by two different reactions give the same results. It appears highly improbable that two completely different sugars should give identical absorption in the same range in two different reactions. The results of determinations of one or two hexoses can be checked by another cysteine reaction. When three hexoses, however, have to be identified in a mixture, we will have to rely for checking on the two older reactions. Disagreement between the results of two different procedures will indicate that an unknown compound which could not be accounted for is present in the unknown solution.

SUMMARY

1. Three characteristic reactions of hexoses with cysteine and H_2SO_4 are described.

2. These reactions permit detection and quantitative determination of hexoses in general, as well as of individual hexoses in mixtures with each other and in the presence of other sugars.

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Studies on the Metabolism of Ribose. V.[1] Enzymatic Conversion of Ribose into Hexosemonophosphate [2]

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INTRODUCTION

In earlier papers (1–4) it was reported that tissue enzymes metabolize D-ribose of purine nucleosides and nucleotides as well as D-ribose-5-phosphate. An aldolase split was suggested (3) to account for the decreased intensity of the orcinol pentose reaction. From incubation mixtures containing liver enzymes and nucleosides, carbohydrate phosphoric acid esters were obtained as barium salts which resembled the ester isolated first by Robison (5) from yeast and later by Embden (6) from tissue. A mixture of glucose-6-phosphate and fructose-6-phosphate could be isolated (3). The experimental details of the isolation will be given here.

EXPERIMENTAL

Substrates and Analytical Procedures

The nucleosides were obtained from yeast ribonucleic acid by the methods of Bredereck (7). They were recrystallized repeatedly and analyzed. Ribose-5-phosphate was prepared as the crystalline barium salt by acid hydrolysis of inosinic acid (8). For pentose determination the orcinol test was used. The heating time in this method should be longer (2,9,10) than specified by Mejbaum (11).

Enzyme Preparation

Our greatest concern was to ascertain that the hexose monophosphate isolated in these experiments was not derived from glycogen or glucose present in the enzyme preparation. Formation of hexose phosphoric acid esters from glycogen in the course of the incubation would cause a decrease of inorganic phosphate in the control samples without added nucleoside. This was not observed and some typical results are listed in Table I. In Exp. 1, crude enzyme, containing phosphatases and phosphoric acid esters, was used. An increase in the concentration of inorganic phosphate was observed in the control experiment (1a), while the esterification of phosphate in the sample containing guanosine (1b) apparently lagged behind the amount of pentose metabolized. However, if the splitting of phosphate in the control experiment is taken into account, the change equals approximately that of the pentose concentration. In Exp. 2, a purified preparation of enzyme was used and the concentration of phosphate remained nearly constant in the control (2a), while in the sample (2b) esterification corresponded to the decrease in pentose concentration.

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[1] Earlier papers of this series: (1–4).

[2] Reported at the Chicago Meeting, American Society of Biological Chemists, May 22nd, 1947.

TABLE I

Degradation of Guanosine and Esterification of Phosphate by Liver Enzymes

Incubation at 37°C.; initial concentration of guanosine and of inorganic phosphate: 10 γ moles/ml. Experimental procedure and enzyme preparation as described earlier (2). For further details see text.

Exp. no.	Substrate	Change in concentration of pentose after 3 hr. incubation	Change in concentration of inorganic phosphate after 3 hr. incubation
		γ moles/ml.	*γ moles/ml*
1a	No substrate	−0.26	+4.45
1b	Guanosine	−8.53	−4.64
2a	No substrate	−0.13	−0.38
2b	Guanosine	−7.6	−7.8

As a source of enzymes we used rabbit liver. This material contained considerable amounts of glycogen and hexose phosphoric acid esters, even when the animals were starved before sacrifice. These carbohydrates, however, were removed in the course of purification of the enzyme preparation. We proceeded as follows:

Three adult rabbits were starved for 24 hr., then killed, and the livers removed. The tissue was homogenized in a Waring Blendor with an excess of acetone, filtered on a Büchner funnel, treated once more with acteone, and dried by a vacuum. The dried powder was suspended in 6 vol. of 0.2 *M* phosphate buffer, pH 7.5, and incubated for 30 min. at 37°C. with occasional stirring. The mixture was centrifuged and the supernatant dialyzed overnight in the icebox against 0.02 *M* phosphate buffer, pH 7.5. The solution was then treated with 2 vol. of satd. $(NH_4)_2SO_4$ solution and the resulting precipitate centrifuged. It was taken up in the original volume of 0.02 *M* buffer and treated again with 2 vol. of satd. $(NH_4)_2SO_4$ solution. The precipitate was centrifuged, dissolved in $\frac{1}{4}$ of the original volume of buffer, and dialyzed overnight against 0.02 *M* phosphate buffer of the pH 7.5. The dialysis was carried out at low temperature under stirring and with 2 changes of the outside liquid. Some denatured protein was removed by filtration. The nitrogen content of this solution was found to range between 0.5 and

1.5 mg./ml. Table II shows how the glycogen content and reducing sugar were decreased by this procedure. The data exclude the enzyme preparation as a possible source of the hexose monophosphate.

TABLE II

Removal of Glycogen and Reducing Sugar in the Course of Purification of Enzymes Concerned with Ribose Metabolism

The fractionation of the enzyme is described in the text. Glycogen was determined following the directions of Colowick (12) and Cori (13); reducing sugar was estimated by the method of Shaffer and Somogyi (14) and calculated as glucose. Robison ester reduces like 60–70% of an equimolar amount of glucose. Identical results were obtained with and without deproteinizing by tungstic acid.

Enzyme fraction	Experiment 1		Experiment 2	
	Glycogen	Reducing sugar	Glycogen	Reducing sugar
	mg./ml.	*mg./ml.*	*mg./ml.*	*mg./ml.*
Crude buffer extract of acetone-treated rabbit liver	0.16	1.43	0.3	3.8
Same, after dialysis			0.2	0.7
Enzyme solution after two $(NH_4)_2SO_4$ precipitations	0	0.5	0	0.1
Same, after final dialysis	0	<0.05	0	<0.1

Preparation and Isolation of Hexose Monophosphate

The following directions for the preparation of hexose monophosphate from guanosine hold also for its isolation from adenosine or ribose-5-phosphate. If adenosine serves as substrate, some purified adenosine deaminase (15) should be added. This enzyme is lost during the purification of the rabbit liver extract. Adenosine must be converted to inosine to make possible the action of nucleosidase (16) which is the first step in the sequence of reactions. Guanosine is split by nucleosidase without preliminary deamination.

Two g. of pure guanosine (7 millimoles) are dissolved by heating in 200 ml. of water and cooled to about 50°C., yielding a supersaturated solution. This is mixed with 500 ml. of purified enzyme extract and incubated for 3 hr. at 37°C. As a control experiment 2.5 ml. of the enzyme extract and 1.0 ml. of water are incubated under identical conditions. At time zero, and after 3 hr., small samples for pentose and phosphoric acid determination are taken in the fashion described earlier (2). A pilot experiment may be arranged prior to the incubation to test the activity of the enzyme extract and to determine the optimum incubation time. In most of our experiments, a period of 3 hr. was found suitable. The reaction is stopped with ⅛ vol. of 50% trichloroacetic acid and the mixture is kept in the refrigerator for at least 1 hr. The precipitate is filtered off on a Büchner funnel and washed with a small amount of 5% trichloroacetic acid. The combined solutions are treated with strong NaOH to give a pH of 7.0–7.5.

Concentrated barium acetate solution is added to precipitate the free phosphate and part of the organic phosphate, which is centrifuged off and washed. The supernatant and wash water are combined and treated with sufficient lead acetate solution to cause complete precipitation. A second lead fraction is obtained by adjusting the pH to 8.5 with NaOH. Since the analytical data for both fractions are usually similar, we combine them for further purification. The precipitates are washed with a small amount of cold water and decomposed with H_2S. The PbS is filtered off and washed carefully several times with small amounts of hot water. H_2S is removed by vacuum distillation and the solution is concentrated to 20–30 ml. Saturated $Ba(OH)_2$ is added to pH 8.5 and the water-insoluble barium fraction is separated. Two to three volumes of cold alcohol are added to the solution, the resulting precipitate is centrifuged down, washed with absolute alcohol, and dried. The average yield of this fraction is 1.2 g. It contains about 5% phosphorus as organic phosphate and 1–2% pentose. The barium salt is purified and converted into the calcium salt following closely the directions of Warburg and Christian (17). In addition to the elementary analysis and analytical data given in our earlier note (3) we would like to give the characteristics of another preparation.

Elementary analysis:[3] $C_6H_{11}O_9PCa + 2H_2O$ (M.W. 334.2). Calculated: 21.35% C, 4.48% H, 9.30% P, 12.0% Ca; Found: 20.15% C, 4.40% H, 9.44% P, 13.1% Ca. Polarimetric determination: $[\alpha]_D = +0.51°$ (0.1 *N* HCl; $l = 2$; $c = 1.047$); $[\alpha]^{26°}_{D} = +24.4°$. Hypoiodite reduction: 1.37 equivalents of iodine per mole of ester (18). Hydrolysis in 1.0 *N* HCl at 100°C.: After 30, 60, and 120 min., 10.1, 20.6, and 28.3% of phosphoric acid were split off. For further characterization of our product the Seliwanoff reaction modification of Roe (19), was carried out. The pigment obtained had absorption maxima at 405 mμ and 480 mμ. The same absorption maxima are shown by the resorcinol reaction product obtained from fructose, but the color intensity of the latter is considerably higher. An authentic specimen of Robison ester from yeast kindly furnished by Dr. E. Haas behaved like our ester. Prolonged heating of the samples did not increase the color intensity significantly.

The analytical data presented here and elsewhere (3) show that the hexose monophosphate isolated by us is a mixture of about 70% aldose and 30% ketose phosphate. This is in agreement with the observations on "equilibrium ester" (20) discovered by Robison (5) and by Embden (6).

For comparison, the following data reported by others are listed:

Polarimetric Determination in Acid. $[\alpha]_D = +29.5°$ (6); $[\alpha]_D = +25.4$ to $+28.9°$ (17). The values depend on the proportion of the components of the mixture. Glucose-6-phosphate shows $[\alpha]_D = +41.4°$; fructose-6-phosphate $[\alpha]_D = +1.5°$ (20).

Hypoiodite Titration. Glucose-6-phosphate reduces 2.0 equivalents of iodine; fructose-6-phosphate does not reduce the hypoiodite reagent (18). Values reported for the natural mixture of the esters range from 1.0 equivalent of iodine (17) to 1.5 equivalents (21).

Hydrolysis by Acid. Lohmann (22) reports for Embden ester: After 30, 60, and 180 min. in 1.0 *N* HCl at 100°C., 13.2, 17.2, and 26.3% phosphoric acid liberated; the corresponding values for Robison ester were 13.2, 17.2, and 27.0% mineralization.

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[3] C and H determination: Arlington Laboratories, Fairfax, Virginia.

DISCUSSION

The conversion of the C_3-fragments of ribose into hexose explains our observations on the change of the orcinol reaction in the course of ribose metabolism. Fig. 1 shows the absorption spectra of the pigments derived from ribose and its esters or nucleosides and from hexose monophosphate.

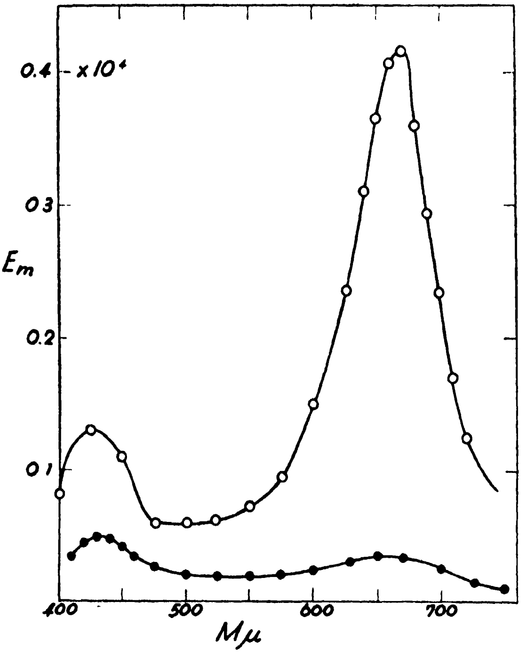

FIG. 1. Absorption spectrum of pigment obtained in the orcinol reaction with ribose derivatives and with hexosemonophosphate. Ribose-5-phosphate: -○-○-○-○-; Robison ester: -●-●-●-●-. The orcinol reagent specified by Mejbaum (11) was used. The sample (0.1 γ mole) was dissolved in 1.5 ml. of H_2O and heated with 1.5 ml. of reagent for 40 min. in a boiling water bath. The volume was brought to 5.0 ml. with water for the measurements in a Beckman spectrophotometer, model D.

Suggestions concerning the sequence of reactions leading from ribose to hexosephosphates have been given in our earlier reports (3,23). A detailed discussion of the relation of this work to the investigations of Klein (24), Kalckar (16), Dickens (25), Dische (26), and Krampitz and Werkman (27) is given elsewhere (28).

SUMMARY

1. One of the reaction products of ribose metabolism has been isolated and identified as hexose monophosphate.

2. The purification of the enzyme preparation from rabbit liver used in these experiments is described.

3. The absorption spectra of the reaction products obtained from ribose and its derivatives and from hexose monophosphate with orcinol and HCl are reported.

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The Metabolism of Niacin in Ruminants (Sheep, Goats and Calves)[1]

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INTRODUCTION

That there is a species difference in the metabolism of nicotinic acid has been shown by a number of studies. The principal end product, as evidenced by urinary excretion, is N′-methylnicotinamide (NMN) in man (1–5), dogs (6), pigs (7), rats (8,9), and cotton rats (10). On the other hand, species of herbivora, such as the guinea pig and rabbit (11), the horse (12), and ox (13), do not excrete appreciable amounts of NMN after the ingestion of nicotinic acid or nicotinamide. It appears that these species either do not methylate nicotinic acid and its amide or, if they do, that they destroy the methylated product as rapidly as it is formed. Recently, it has been shown that man excretes large amounts of N′-methyl-6-pyridone-3-carboxylamide on normal diets and following the ingestion of nicotinamide (14,15), while the rat excretes only a small amount of this compound.

From the standpoint of comparative biochemistry it was of interest to study the metabolism of nicotinic acid in polygastric herbivora as, except for work with calves (13), this had been studied in only monogastric herbivorous animals. For these studies goats, sheep and calves were used.

EXPERIMENTAL

The sheep and Angora goats used were mature animals. They were fed a standard diet of hay and grain. The calves were between the ages of 1 and 2 months. Their diet

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consisted of skim milk, hay and a concentrate mixture typical of that normally fed to growing dairy calves. The urine was collected quantitatively by means of a specially constructed metabolism cage. Toluene was used to prevent bacterial growth. The first day's collection for each period was stored in the refrigerator and then mixed with the second day's collection. Each animal was subjected to 3 different regimens. The urine was collected for 2 consecutive days while the animal was on a stock diet. Two g. of nicotinamide were then administered in a gelatin capsule *per os* on each of 3 consecutive days. The urine was collected on the 2nd and 3rd days. After a week had elapsed, 2 g. of nicotinamide dissolved in 10 ml. of water were administered by subcutaneous injection for each of 3 consecutive days, and the urine collected on the 2nd and 3rd days.

Nicotinic acid was determined by the microbiological method (16) on the 2-day composite urines. The urines were then brought to a pH of approximately 4, toluene added and shipped to Duke University for analyses of N′-methylnicotinamide and N′-methyl-6-pyridone-3-carboxylamide (pyridone). The NMN was determined by the method of Huff and Perlzweig (17), and pyridone by the method of Rosen, Perlzweig and Handler (15). The essential features of the modification consisted of clearing the urine with lead subacetate and extracting the filtrate with ether after saturating with solid K_2CO_3 (18).

RESULTS AND DISCUSSION

The urinary excretion of nicotinic acid, NMN and its pyridone is shown in Table I. Following the administration of nicotinamide there was a substantial increase in the amount of non-methylated nicotinic acid derivatives excreted in the urine. The sheep and calves excreted by the renal pathway between 14 and 19% of the amount ingested, measured as nicotinic acid. The goats excreted 23% when the administration was subcutaneous, and only 6.4% when the nicotinamide was ingested. Following the ingestion or subcutaneous administration of nicotinamide there was a small but insignificant increase in the amount of NMN excreted. In this respect, these 3 species behave in a manner similar to that observed in the rabbit, guinea pig, and horse. The data on the excretion of NMN by calves are in accord with previous observations (13) that there is no significant difference in the excretion of NMN by calves fed a nicotinic acid-free diet and those receiving a supplement of nicotinic acid.

On a normal diet, none of the 3 species studied excrete measurable amounts (less than 2 mg./day) of N′-methyl-6-pyridone-3-carboxylamide. The increments over the basal value of pyridone excreted by sheep and goats, while small, appear to be real. It is apparent that pyridone is not an end product of the metabolism of nicotinamide in the calf, and that it is of minor quantitative importance in the two other species.

TABLE I

Daily Excretion of Nicotinic Acid, N'-Methylnicotinamide and N'-Methyl-6-Pyridone-3-Carboxylamide by Sheep, Goats, and Calves

Animal	Body wt.	Basal diet			2 g. Nicotinamide daily					
					Ingested			Subcutaneously		
		NA	NMN	Pyridone	NA	NMN	Pyridone	NA	NMN	Pyridone
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Sheep 46	46	3.0	2.8	0[a]	224	3.4	3.8	259	6.8	0
47	28	2.3	1.4	0	400	3.2	0	350	3.8	7.6
49	61	8.0	1.1	0	226	7.5	5.0	261	9.5	4.9[b]
Goat 10	20	5.1	1.7	0	66	2.1	7.5	807	4.1	2.3[b]
20	29	6.3	2.7	0	85	7.8	6.2	286	6.0	8.5
30	24	1.1	1.4	0	231	4.2	2.0	286	2.7	2.6
Calf 40	35	1.7	3.7	0	490	7.4	0	388	4.7	0
41	31	6.9	6.4	0	320	5.0	0	520	6.5	0
72	66	10.6	6.5	0	252	6.7	0	241	10.6	0

[a] All 0 values for the pyridone in this table represent figures of less than 2 mg.

[b] Values questionable due to low concentration.

It is of interest to compare the excretion of pyridone by the rabbit and guinea pig with that of the sheep, goat and calf. Rabbits and guinea pigs show a small but definite, and a more pronounced increment in the excretion of pyridone following the ingestion of nicotinmade than do any of the 3 species studied (unpublished data, Rosen and Perlzweig).

These findings do not preclude the possibility of the 2 methylated derivatives being intermediate products in the metabolism of niacin in the herbivora. Thus, in the previous studies on the horse (12), it was shown that, while this species does not excrete significant amounts of NMN after oral or parenteral administration of niacin, when 796 mg. of NMN itself was administered orally, it was largely "destroyed" (only 1% in the urine unchanged). Substantially the same behavior in regard to NMN was observed in the guinea pig, while the rabbit is apparently capable of oxidizing a fair amount of NMN to the pyridone (unpublished data of Rosen and Perlzweig). It is interesting to recall in this connection that Knox, in describing the enzyme capable of oxidizing NMN (14), noted its presence in rabbit liver but not in sheep liver, and that minced sheep liver brei can inactivate the enzyme of rabbit liver.

The data comparing the metabolism of niacin in man, dog, rat, and herbivora will be presented in a separate communication (19).

SUMMARY

After oral and subcutaneous administration of 2 g. nicotinamide daily for 3 days, goats, sheep, and calves show a slight but insignificant increase in the renal excretion of N′-methylnicotinamide. Goats and sheep showed a slight increase in the excretion of N′-methyl-6-pyridone-3-carboxylamide, while there was no measurable amount excreted by the calf.

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The Effect of Long-Chain Carbon Compounds, Particularly Hydrocarbons, on the Metabolism of Tubercle Bacilli

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INTRODUCTION

The utilization of hydrocarbons as energy source by numerous genera of bacteria is a well established fact. The literature on the subject has been adequately reviewed by ZoBell (1). The *Mycobacteria* have been among those organisms particularly implicated in the bacterial degradation of hydrocarbons. Söhngen (2), as early as 1913, showed that at least 6 species of soil *Mycobacteria* were able to attack paraffin wax as well as other hydrocarbons. Others (3,4,5,6) have reported that nonpathogenic *Mycobacteria* from various sources are able to assimilate and decompose various pure or impure hydrocarbons, aromatic as well as aliphatic. Haas (7) reported that *Mycobacterium leprae, M. phlei* and *M. smegmatis* oxidized both light and heavy petroleum oils and paraffin wax, but his attempts to grow mammalian tubercle bacilli in hydrocarbon media failed, although an avian strain grew slowly on paraffin. Gordon (8) stated that many of the *Mycobacteria* isolated from infected animal tissue utilized paraffin, and in other ways appear to be identical with soil strains. However, the possibility that these organisms were merely soil contaminants cannot be overlooked (9).

The effect of long-chain, aliphatic fatty acids on the growth and metabolism of *Mycobacteria* has been the subject of various investigations (10,11,12). Dubos (13), and Dubos and Davis (14), have recently studied the effect of oleic acid on the growth of these organisms in some detail. Since some investigators have claimed that fatty acids are intermediate or end-products of hydrocarbon oxidation by bacteria, it was thought of interest to determine the effect of long-chain hydrocarbons on the oxidative metabolism and growth of representative strains of pathogenic Mycobacteria.

EXPERIMENTAL

Materials and Methods

Four strains of pathogenic *Mycobacteria* were used in this investigation, the H37Rv (virulent) and H37Ra (avirulent), obtained from Mr. W. Steenken, Trudeau Laboratories, Saranac, N. Y.; the Sigerson (avian) obtained from Dr. W. Feldman, Mayo Clinic, and a BCG strain.

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For oxidative metabolic studies, the organisms were grown in Tween 80—bovine serum fraction V (albumin) medium (15) distributed in 100 ml. amounts in 500 ml. cotton-plugged Erlenmeyer flasks. Each flask was inoculated with 4 ml. of a 7–10 day old culture of the appropriate organism and incubated at 37.5°C. for 7–10 days. At the end of this period, 80–90 ml. of supernatant fluid were withdrawn from each flask by a pipette. The remaining fluid, with the organisms, was transferred to 250 ml. centrifuge bottles and centrifuged at 2000 r.p.m. for 20 min. The supernatant fluid was then withdrawn by pipette and the bacterial deposit was resuspended in 30 ml. of $M/15$ phosphate buffer, pH 6.8 or 7.4, and recentrifuged. This washing procedure was performed twice. The organisms were then kept in the ice chest at 5°C. until needed. The H37Ra and Sigerson strains could be harvested and kept at ice chest temperature for 5–7 days without deterioration, the H37Rv and BCG strains slowly lost ability to oxidize various substrates when left for periods longer than 72 hr.

Immediately prior to use in oxidative studies, the organisms were resuspended in such amounts of $M/15$ phosphate buffer, pH 6.8 or 7.4, that each ml. of suspension contained 13–17 mg. of bacterial cells on a dry weight basis. Dry weights were determined in the following manner:

Forty ml. of a 7–10 day culture of organisms were transferred to a 100 ml. centrifuge tube and centrifuged at 4500 r.p.m. for 1.5 hr. The supernatant fluid was then withdrawn by pipette and the organisms, resuspended in 5 ml. of $M/15$ phosphate buffer, pH 7.4, containing 0.1% Tween 80, were transferred quantitatively by means of capillary pipette, to tared Wassermann tubes. The organisms were again centrifuged as above, resuspended and washed twice with $M/15$ phosphate buffer, pH 7.4, and finally with 7 ml. of 95% ethyl alcohol. After pipetting off the alcohol, the organisms in the Wassermann tubes were placed in a vacuum oven at 65°C. for 24 hr., and the weight of the organisms calculated by difference. At the same time ,the original culture was serially diluted and the optical density of each dilution determined in the Coleman Junior Clinical Spectrophotometer. A straight line relationship was obtained when optical density of the diluted culture was plotted against dry weight of organisms. After this calibration, optical densities were determined on each culture, and dry weights calculated from the densities.

This procedure was performed with each of the 4 cultures sued. The curve for the Sigerson strain was virtually superimposable upon the BCG curve. It was found that the blank used in the Coleman reading could be either culture filtrate, fresh culture medium, or phosphate buffer, with no significant difference in result at a wavelength of 650 mμ.

Oxygen uptake was measured in the Warburg apparatus, under air, at 37.5°C. in the usual manner. There was added to each Warburg vessel, 1.0 ml. of bacterial suspension 0.05 ml. of hydrocarbon and 0.95 ml. of $M/15$ phosphate buffer, pH 6.8 or 7.4. *n*-Octadecane, which is solid at room temperature, but liquid at 37.5°C., was added to flasks in the melted state. The sidearms contained 0.5 ml. of buffer and the insert 0.2 ml. of 10% KOH, with folded analytical filter paper. Control vessels containing no hydrocarbon received an additional 0.05 ml. of buffer. CO_2 determinations were performed according to the three flask method described by Umbreit *et al.* (16).

The vessels were allowed to equilibrate for 15 min with the manometers open to air. The shaking speed was 120–130 excursions/min. and the amplitude was 3 cm. Slower shaking and smaller amplitudes decreased the O_2 uptake slightly. Higher shaking speeds did not alter the results. In general, reactions were allowed to proceed for 5 hr.

All the chemically defined hydrocarbons were purchased from the Connecticut Hard Rubber Company, New Haven, and were guaranteed to be at least 95 mole-% pure.

RESULTS

Effect of Saturated and Unsaturated Hydrocarbons on O_2 Consumption

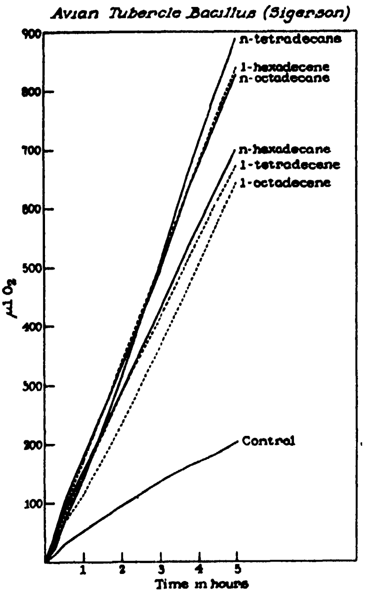
It was noted that, with all hydrocarbons of chain length greater than 8 carbons, the organisms migrated almost completely to the buffer-hydrocarbon interface, leaving only a slightly turbid aqueous layer. In the case of the octenes and octane, the organisms clumped into a large, pea-sized, yellowish floating mass of rubbery consistency. Hydrocarbons containing less than 8 carbons did not exhibit this effect. The migration of acid-fast bacilli to the hydrophobic layer has been studied in detail by Reed and Rice (17), among others.

In general, the Sigerson (avian) strain showed considerably greater increases in O_2 uptake under the influence of various hydrocarbons than did the other 3 strains studied. However, the 14, 16 and 18 carbon saturated and 1-unsaturated hydrocarbons greatly increased the O_2 consumption of all 4 strains of organism. Figs. 1–4, which are representative experiments, show the extent of this effect.

With *n*-decane and *n*-dodecane, the BCG, Sigerson and H37Ra strains showed definite, increased O_2 uptake, though somewhat less than with the higher hydrocarbons. On the other hand, the H37Rv strain, respiring in the presence of the 10 and 12 carbon saturated compounds, showed slight or no increase in O_2 consumption.

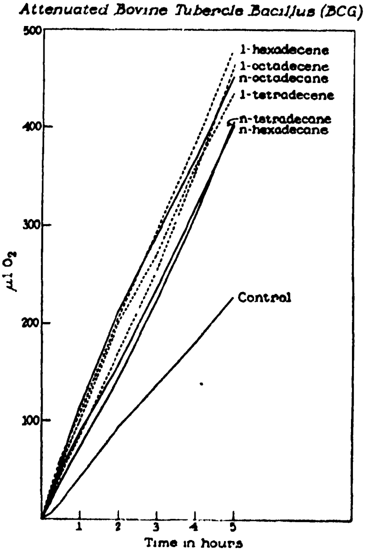
In the presence of 1-dodecene, H37Ra respired as well as in the presence of the higher saturated and unsaturated homologs, while the avian strain showed a somewhat smaller increase in O_2 consumption. Both the BCG and H37Rv strains were inhibited by 1-decene and 1-dodecene. The degree of stimulation of the H37Ra and Sigerson strains by 1-decene was considerably less marked than with the higher homologs.

n-Octane caused either slight inhibition or slight stimulation of the respiration of all 4 strains, whereas 1-octene and 2-octene and 1-heptene markedly inhibited the respiration of all organisms. It is possible that the effect of the 8 carbon compounds may be due to the clumping of the organisms in the experimental flasks.



Avian Tubercle Bacillus (Sigerson)

FIG. 1. Effect of 40 mg. of various hydrocarbons on O_2 uptake of avain tubercle bacillus.



Attenuated Bovine Tubercle Bacillus (BCG)

FIG. 2. Effect of 40 mg. of various hydrocarbons on O_2 uptake of BCG strain of tubercle bacillus.

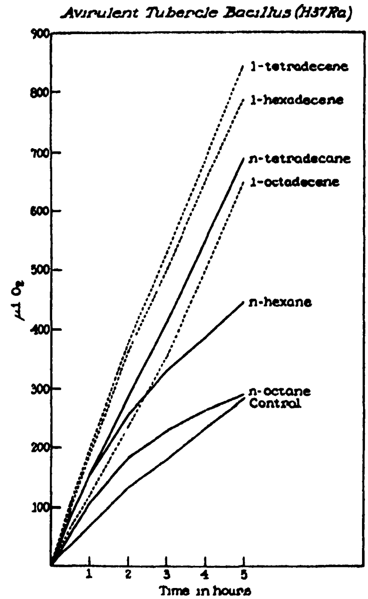


FIG. 3. Effect of 40 mg. of various hydrocarbons on O_2 uptake of H37Ra strain of tubercle bacillus.

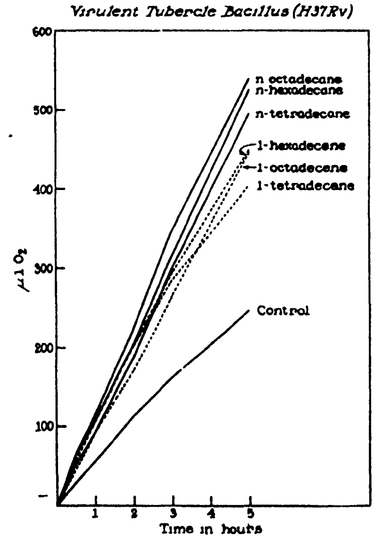


FIG. 4. Effect of 40 mg. of various hydrocarbons on O_2 uptake of H37Rv strain of tubercle bacillus.

When respiring in the presence of *n*-hexane, the BCG strain exhibited marked stimulation, whereas the other 3 strains were, for the most part, unaffected. 1-Hexene had no effect on the respiration of any of the strains.

The above results are summarized in Table I. It should be mentioned that the hydrocarbons alone, or hydrocarbon plus heat-killed tubercle bacilli showed no O_2 uptake.

TABLE I

Effect of Various Hydrocarbons on the Respiration of Washed Mycobacteria

	Hydrocarbons																
	Control no hydrocarbon	*n*-Hexane	*n*-Octane	*n*-Decane	*n*-Dodecane	*n*-Tetradecane	*n*-Hexadecane	*n*-Octadecane	1-Hexene	1-Heptene	1-Octene	2-Octene	1-Decene	1-Dodecene	1-Tetradecene	1-Hexadecene	1-Octadecene
Sigerson	−200	−231	−243	−616	−650	−720	−700	−706	−238	−100	−143	−29	−300	−365	−571	−600	−580
H37Ra	−280	−380	−257	−373	−512	−597	−562	−595	−388	−180	−215	−160	−398	−550	−659	−722	−561
BCG	−264	−374	−230	−317	−366	−440	−487	−485	−304	−126	−120	−78	−249	−184	−371	−440	−440
H37Rv	−266	−301	−226	−329	−322	−540	−545	−565	−274	−241	−151	−45	−239	−189	−377	−435	−499

The figures given are the average of O_2 uptake in µl. of numerous experiments. The duration of the experiments was 5 hr. Each cup contained 1 ml. bacterial suspension. 0.95 ml. $M/15$ PO_4 buffer, pH 6.8 or 7.4, and 0.2 ml. 10% KOH in well.

Effects of Miscellaneous Hydrocarbons on Oxidative Metabolism

Paraffin wax slightly increased the respiration of all 4 strains, the BCG strain being least affected. Ligroin (B.P. 60°–90°), *n*-heptane (B.P. 09°–100°), petroleum ether (B.P. 40°–50°), and Squibb Heavy Mineral Oil, either had no effect or decreased the O_2 uptake. Xylene and toluene completely inhibited the O_2 uptake of all strains.

Determination of RQ

n-Tetradecane was the substrate used for determination of the RQ. The theoretical RQ for a hydrocarbon completely oxidized to CO_2 and H_2O is 0.65. RQ values of 0.83 and 0.72 were noted for the BCG and H37Ra strains, respectively. If the hydrocarbon is being oxidized, this would suggest that intracellular decarboxylation mechanisms are being stimulated as well. The H37Rv strain gives an RQ of 0.67, a value indicating complete oxidation of the substrate, while the Sigerson strain gives an RQ value of 0.48, indicating approximately 75% oxidation of the substrate. Endogenous RQ measurements varied from 0.92 to 1.0 for all strains. It is realized that no definitive information as to the course of matebolism can be secured from RQ values alone.

Effect of Two Long-Chain Alcohols on the Oxidative Metabolism

Oxygen uptake of the 4 strains was markedly stimulated by both cetyl and stearyl alcohols when flakes of these compounds were added to Warburg vessels. The O_2 uptake was considerably greater with cetyl alcohol than with stearyl alcohol, the Q_{O_2} being tripled in many cases.

Attempts to Isolate Acids as End Products of Hydrocarbon Metabolism

After the bacteria had acted upon *n*-tetradecane for 5 hr., the suspension of organisms was centrifuged for 1 hr. at 4500 r.p.m. The supernatant was removed and made acid (pH 3) with 10 N H_2SO_4. An aliquot portion was placed in the Kirk all-glass micro steam distillation apparatus. Five 10 ml. portions of distillate were collected. Titration with 0.01 N NaOH showed no acid present in the distillate.

Acidification of the reaction supernatant with 10 N H_2SO_4 and extraction wth three 3 ml. portions of ether yielded no ether-soluble acids.

Attempts to Grow Tubercle Bacilli in Media Containing Hydrocarbon as Energy Source

A basal medium containing the salt composition suggested by Dubos and Middlebrook (15) was used. This was supplemented by 0.1% NH_4NO_3 or $(NH_4)_2SO_4$. The medium was dispensed in 5 ml. amounts into metal-capped potato tubes and autoclaved at 15 lb. pressure for 15 min. The hydrocarbons used, 14–18 C saturated and unsaturated, were autoclaved separately at 10/10 and added aseptically in 0.3 ml. amounts to the tubes. Tubes were inoculated with a 2×10^{-4} dilution of a 7 day old Tween 80—albumin culture and incubated at 37.5°C.

No growth was obtained in the basal medium + hydrocarbon, even after 4 weeks incubation. Two-tenths per cent asparagin was then added to the medium in place of the ammonium salts.This basal-asparagin medium supported satisfactory minimal growth.

With the Sigerson strain of tubercle bacilli, coarse, granular bottom growth appeared in 2 days and increased in amount at least up to 6 weeks in tubes containing *n*-tetra- and hexadecane and 1-octadecene. On the other hand, growth in the asparagin-basal medium without hydrocarbon appeared only after 6 days and remained minimal throughout the 6 weeks of the experiment.

No growth was obtained using *n*-octadecane and 1-tetra- and hexadecene.

Similar experiments with the H37Ra strain failed. No growth was obtained in any of the tubes containing hydrocarbons, though there was minimal growth in the control tubes.

Since the possibility existed that the hydrocarbons were dissimilated to long-chain fatty acids, and since serum albumin is known to neutralize the toxicity of higher fatty acids for *Mycobacteria,* bovine albumin (serum fraction V) was added aseptically in a final concentration of 0.5% to the asparagin-containing control medium. When hydrocarbons were added to this medium, good growth occurred with *n*-tetra-, hexa- and octadecane, but growth was no better than in the basal-asparagin-albumin medium. No growth was obtained in media containing the unsaturated 14–18 carbon hydrocarbons.

It would thus appear that, with the system used, the hydrocarbons tested do not support the growth of the H37Ra strain of tubercle bacillus. Similar results were obtained with the H37Rv strain.

DISCUSSION

It is obvious from the foregoing that certain hydrocarbons exert a profound effect upon the oxidative metabolism of tubercle bacilli. In many cases, apparently the respiration of the *Mycobacteria* studied is more stimulated by hydrocarbons than by any of the more usual carbon sources. The increase in O_2 consumption due to the 14, 16, and 18 carbon hydrocarbons is much larger than the increase seen, for example, with glucose or glycerine.

There are several possible explanations for the stimulation observed. Most obvious is the possibility that the hydrocarbons are utilized as energy sources by the organisms. R.Q. measurements and the fact that no end-products (*e. g.*, aldehydes, acids) have been found in the reaction mixture after 5 hr. of incubation would tend to indicate that the compounds are almost completely oxidized. However, only in the case of the Sigerson (avian strain) has stimulation of growth been noted in the presence of hydrocarbons.

A clear-cut answer to the question of actual utilization could presumably be supplied by the use of hydrocarbons containing isotopic carbon and measurement of the liberated CO_2 for isotope content. Work along these lines is being done.

It is quite possible that the results observed are caused by the dispersing or congregating effects of the hydrocarbons on the bacteria, thus increasing or decreasing the respiration by changing the total free surface of the cells. Lending credence to this hypothesis is the fact that, in the case of the 8-carbon compounds, which inhibit respiration, a marked congregating action of the hydrocarbons is observed. On the other hand, those hydrocarbons which stimulate the respiration quite obviously wet the surface of the bacteria, and presumably allow for dispersion.

These possibilities have been presented as being most likely to account for the mode of action of the hydrocarbons. Undoubtedly there are others, and further work is being done to elucidate the mechanism of the effect.

SUMMARY

1. Higher hydrocarbons and alcohols have been tested for their effect on the oxygen uptake of tubercle bacilli. The 14, 16, and 18 carbon saturated and unsaturated hydrocarbons, in particular, greatly increase the O_2 uptake of 4 strains of pathogenic mycobacteria studied. Cetyl and stearyl alcohols have an anlogous effect. Fatty acids are apparently not end-products of the reaction.

2. *n*-Tetradecane, *n*-hexadecane and 1-octadecene stimulate the growth of the avian strain. The other strains tested are apparently unable to utilize the hydrocarbons as energy source for growth.

3. RQ measurements are compatible with the hypothesis that hydrocarbons are almost completely oxidized to CO_2 and H_2O. Other possible modes of action of the hydrocarbons are discussed.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. Rene J. Dubos, in whose laboratory this work was performed, for his many valued suggestions and criticisms.

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The Effect of Dietary Protein Restriction on the Coenzyme A Content of Rat Liver [1]

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INTRODUCTION

Lipmann and his coworkers (1) have recently described a new cofactor, coenzyme A, which is essential for the acetylation of sulfanilamide in liver, and of choline in brain. These authors also reported that the coenzyme contained a high content of pantothenic acid, and Olson and Kaplan (2) have shown that the concentration of coenzyme A in the liver, adrenals, kidneys, and heart is decreased in rats maintained on a pantothenic acid-deficient diet.

In a recent publication from this laboratory (3) evidence was presented indicating that the concentrations of nicotinic acid and riboflavin are related directly to the level of liver protein. Since these vitamins are known to be components of coenzymes, the present investigation was undertaken to determine whether the concentration of coenzyme A in the liver is related to the liver protein when the intake of pantothenic acid is not made the limiting factor. For this purpose rats receiving adequate pantothenic acid were depleted of tissue protein by dietary protein restriction and the concentrations of coenzyme A and protein in the liver were compared.

EXPERIMENTAL

Fifteen female Wistar strain rats between 17 and 20 weeks of age, and weighing between 195 and 230 g. were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this period, 7 of the animals were placed on a protein-free diet for a period of 21 days. The remaining 8 animals served as controls and were continued on the normal diet for the 21 day period. Their food consumption, however, was restricted to the average intake of the animals in the experimental group. The normal diet consisted of commercial casein, 24%; glucose, 63%; corn oil, 8%; Salts

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[1] Aided by a grant from Wyeth, Inc., Philadelphia, Pa.

4 (4), 4%; and dried brewers' yeast, 1%. The protein-free diet was similar in composition except that the casein was replaced by an equal amount of glucose. The following vitamins were incorporated into each 100 g. of diet in the amounts indicated: thiamine 0.2 mg., nicotinic acid 2.5 mg., riboflavin 0.3 mg., calcium pantothenate 4 mg., pyridoxine hydrochloride 0.5 mg., inositol 10 mg., choline chloride 100 mg., and 2-methylnaphthoquinone 1 mg. Twice weekly each animal received 2 drops of a cod liver oil-vitamin E supplement which furnished 200 U. S. P. units of vitamin A, 20 U. S. P. units of vitamin D, and 1 mg. of α-tocopherol.

At the end of the feeding period the animal was sacrificed by a sharp blow on the head; its liver was immediately excised and blotted free of adhering blood. In all cases the left lateral lobe was quickly chilled to 0°C. and used for the determination of coenzyme A. The remainder of the liver was sampled for the determination of water, fat, and nitrogen. Water was determined by drying to constant weight at 103°C., and fat by weight difference after extraction with ether. Nitrogen was determined on the dry, fat-free residue by the micro Kjeldahl method. Coenzyme A was determined on the chilled portion of liver by the method of Kaplan and Lipmann (5).

RESULTS AND DISCUSSION

Throughout the course of the study the experimental animals consumed an average of 12 g. of food per day and, on the average, lost 12 g. in body weight per week. The control animals, the food intake of which was restricted to that of the protein-deficient group, consumed an equal amount of food and, on the average, gained 3 g. in body weight per week.

TABLE I

Water, Fat, Nitrogen, and Coenzyme A Contents of Livers from Protein-Deficient and Control Rats

Diet	No of animals	Water	Fat	Nitrogen	Coenzyme A	
					Units[a]/g.	Units[a]/g. N
		g./kg.	*g./kg.*	*mg./g.*		
Protein-free	7	686 (653–714)	84.8 (55–129)	24.4 (21–28)	129.4 (95–153)	5310 (3822–5790)
Control	8	696 (692–704)	40.4 (25–61)	34.1 (32–37)	189.1 (170–208)	5543 (5120–5945)

Figures in parentheses represent ranges of values.

[a] A unit, as used here, is identical with that defined by Kaplan and Lipmann (5), and is that amount of coenzyme A necessary to produce one-half the maximum amount of acetylated sulfanilamide which can be produced using the test system described by these authors.

The results obtained from the analyses of the livers of the protein-deficient and control groups of rats are presented in Table I. It can be seen that the rats maintained on the protein-free diet exhibited a decrease of the water and nitrogen and an increase of the fat contents of their livers as compared to the control animals. There was also a decrease in the coenzyme A concentration/g. of liver. However, calculated on the basis of liver nitrogen, the coenzyme values of the protein-deficient and control groups showed no appreciable difference.

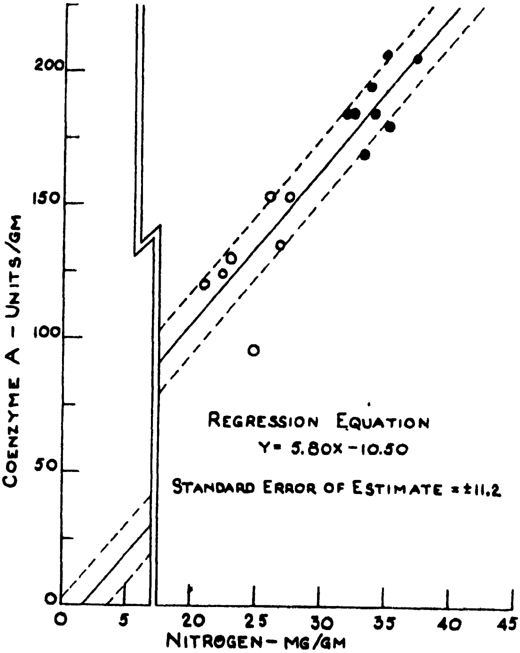

Fig. 1. Liver coenzyme A concentration with respect to variations in liver nitrogen content. Circles represent values obtained with animals on the protein-free diet; solid points represent values obtained with control animals. Data are expressed on a fresh-weight basis.

From an inspection of the data presented in Fig. 1, it will be noted that the concentration of coenzyme A in the liver was directly proportional to the concentration of nitrogen in the liver. That the rate of change in the coenzyme concentration was apparently the same as the rate of change in the nitrogen concentration is evidenced by the fact that the line, when extrapolated to a coenzyme concentration of zero, very nearly passes through the origin. This is in contrast to the rate of change of the liver concentrations of riboflavin and nicotinic acid with respect to liver nitrogen as reported previously (3). In the latter instance there was, apparently, a more rapid decrease in the levels of these vitamins than in the level of nitrogen.

It is of interset to note that the decrease in the concentration of coenzyme A in the liver which accompanied dietary protein restriction occurred even though the daily intake of pantothenic acid was approximately twice the amount usually allowed for adequate diets. Thus, it appears that, in addition to an adequate intake of pantothenic acid, the rat requires protein in the diet for the normal storage of this vitamin in the liver as coenzyme A.

SUMMARY

The effect of feeding a diet free of protein, but adequate with respect to pantothenic acid, to rats for a period of 21 days has been studied with regard to the liver coenzyme A concentration.

The maintenance of rats on such a régime was accompanied by a decrease in the liver water, nitrogen, and coenzyme A, and an increase in the liver fat.

The concentration of coenzyme A in the liver was found to be directly proportional to the level of liver nitrogen. The rate of decrease in the coenzyme A concentration was equal to the rate of decrease in the nitrogen under the conditions employed in this study.

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Two Antifungal Substances from *Bacillus subtilis* Cultures

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INTRODUCTION

Bacillus subtilis (A.T.C.C. 6633) has been cultured by a variety of methods at the Western Regional Research Laboratory for the production of the antibiotic, subtilin (1,2,3,4), which is active principally against certain bacteria (5). The work described here has shown that such cultures produce other antibiotics which are active against fungi.

METHODS

Culture of B. subtilis

Cultures were grown for subtilin production by the following methods: asparagus juice medium, surface culture (2); beet molasses medium, surface culture (2); asparagus juice medium, submerged culture (3); semi-synthetic medium, submerged culture (6). All of these culture methods produced antifungal activity as described below. Concentrates of the antifungal substances were prepared from submerged cultures grown in a large fermenter on a semi-synthetic medium (6).

It was assumed that the antifungal substances would be a byproduct of subtilin production. Therefore, no attempt was made to increase antifungal activity by deviation from culture techniques known to produce a high yield of subtilin.

Antifungal Assay

Petri plates containing the antibiotic preparation in varying concentrations were inoculated with agar plaques cut with a sterile cork borer from plate cultures of the test fungus. After incubation, the diameter of the resulting circular colony was taken as a measure of growth.

Two fungi, *Rhizoctonia solani* and *Aspergillus niger*, were selected for routine assays because of their differential response to different fractions, and because of their rapid growth and ease of manipulation. The final products were assayed against the 12 fungi listed in Table I.

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[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

TABLE I

Antibiotic Spectra of Rhizoctonia and Aspergillus Factors

	Activity against fungi[a] (concentration giving 50% inhibition) p. p. m.	
	Rhizoctonia factor	Aspergillus factor
Pythium sp.	270	very little inhibition
Rhizopus nigricans	160	8.1
Rhizoctonia solani	45	4.0
Sclerotium bataticola	64	3.5
Sclerotinia fructicola	14	5.9
Fusarium lycopersici	no inhibition	85
Penicillium digitatum	68	6.2
Aspergillus niger	>1000	9.9
Aspergillus oryzae	>1000	19
Botrytis cinerea	6.4	4.8
Alternaria citri	470	27
	Activity against yeasts (concentration giving at least 95% inhibition) p. p. m.	
Candida chalmersi castellani	>900	33
Zygosaccharomyces mandschuricus	33	33
Debaryomyces membranaefaciens	>900	33
Hanseniaspora melligeri	>900	33
Torulopsis dattila	>900	>100
Hansenula anomala	>900	22
Saccharomyces cerevisiae	900	11
	Activity against a bacterium (concentration giving 50% inhibition) p. p. m.	
Micrococcus conglomeratus	>180	>13

[a] Assay medium for fungi and yeasts was potato dextrose agar, as discussed under "Antifungal assay." Observed potencies against *R. solani* were higher than on the synthetic assay medium used in preparation of the concentrate.

Before further treatment, *B. subtilis* culture samples were steamed for 10 min. at pH 2.5 to kill the spores, which otherwise interfered with the bioassay. They were not handled aseptically thereafter. This treatment was known not to affect their antifungal potency.

The samples to be assayed were brought to pH 6.0 and added in appropriate amounts to an agar medium of the same pH. Before plating, the mixture was held for 20 min. in a water bath at 60°C. This was found to reduce fungal contaminations to the point where they did not interfere with the assays. Bacterial contaminants were rarely seen, in spite of the use of nonsterile samples, perhaps because the relatively low pH prevented rapid growth.

A medium was necessary which would give approximately optimal growth of the test fungus. Otherwise, growth was stimulated by impurities in some preparations. The medium used for assays with *R. solani* and *A. niger* contained the following ingredients (in g./l.): sucrose, 70; dextrose, 10; yeast extract, 10; glutamic acid, 5; KH_2PO_4 5; $MgSO_4 \cdot 7H_2O$, 1.5; agar, 15. The following trace elements (in p.p.m.) were added: Fe, 20; Mn, 20; Cu, 2; Zn, 10; Ca, 50. The pH was 6.0.

Of the test fungi used for assay of the final preparations (Table I), a few would not grow well on the medium described above. Since these preparations were sufficiently purified to eliminate growth-stimulating substances, potato dextrose agar was used for the assays. It was prepared as follows: 250 g. of diced potato/l. of distilled water was heated for 1 hr. at 60°C., strained through cheesecloth, and autoclaved for 30 min.; 2% of glucose and 1.5% agar were added, and the pH adjusted to 6.0.

To obtain uniform inoculum, it was essential to standardize the age of the inoculum cultures. It was also essential to take the disks of inoculum only at a uniform distance from the edge of the colony, and only from typical colonies. For abundant sporebearers, such as *Aspergillus*, inoculum was taken from near the edge of the culture where the mycelium had not yet formed spores. This avoided scattering of the spores.

Maximum precision was attained if plates were inoculated in the center with one organism and incubated until the control had nearly reached the edge of the plate. At the incubation temperature (28°C.) this required 1–3 days, varying with the species. Satisfactory results were obtained, however, when plates were inoculated with 2 or 3 organisms and the respective colonies measured before they had grown together. In this case, it was necessary that the organisms on one plate have approximately the same growth rate. In all cases plates were prepared in duplicate.

After measurement of diameters of the colonies in an assay, the percentage of maximal growth was plotted on logarithmic probability paper (7,8) against the concentration of the antibiotic preparation. From the resulting curve, the concentration of antibiotic which would have given 50% inhibition of growth was determined and taken as the measure of the potency of the preparation. Two or three independent assays on the same preparation did not usually deviate from their average by more than 20%. Comparison with a standard reference material included in each set of assays did not improve the reproducibility of the method.

Antibiotics have also been assayed against fungi by a cup plate method (9). Under our conditions, the edges of the clear areas were poorly defined and very transitory; hence, this method was not used.

The final products were also assayed by a streak assay against 7 yeasts, and turbidimetrically (8) against the bacterium *Micrococcus conglomeratus*.

EXPERIMENTAL

Existence of Antifungal Factors

Whole *B. subtilis* cultures showed antibiotic activity against 11 out of 12 fungi and 4 out of 7 yeasts. Since purified subtilin had no activity against these organisms, it was evident that the antifungal activity of the whole cultures was not due to subtilin but to another substance or substances, which were discarded during the purification process.

Separation of Antifungal Factors from Subtilin

The process of subtilin purification has been described (10). When the freshly harvested culture was extracted with butanol, the extracted culture always contained over half of the original activity against *R. solani*, but it was never active against *A. niger*. This activity was termed "rhizoctonia factor." The butanol extract was then dehydrated by the addition of NaCl. The resulting precipitate of crude subtilin was inactive against the test fungi, and the anhydrous butanol contained all of the original activity against *A. niger* and was slightly active against *R. solani*. The activity in this fraction was termed "aspergillus factor." Removal of crude subtilin from the butanol was also accomplished by two other methods (10), and in each case the crude subtilin lacked antifungal activity, while the remainder of the butanol extract contained the aspergillus factor.

Concentration of Rhizoctonia Factor

When the cells were centrifuged out of the butanol-extracted culture, the antifungal activity remained in the supernatant, which was then adjusted to pH 6, although the pH was not critical within the range of 3 to 7. To 6 l. of supernatant (representing 6.25 l. of original culture), 4 l. of a saturated basic lead acetate solution were added slowly with stirring. The resulting precipitate was separated and suspended, with stirring, in 3.25 l. of aqueous 2.4% $Na_2HPO_4 \cdot 7H_2O$, which gave a slight excess of phosphate by the benzidine blue spot test (11). The solution, which then contained over half of the original activity, was separated from the precipitate, frozen, and dried *in vacuo*, yielding 75 g. of dry supernatant.

The first step in this procedure yielded a very large precipitate, because the lead precipitated sulfates and phosphates from the medium. A reduction in the amount of lead added caused a reduction in the amount of activity precipitated. Recovery of the activity from the lead precipitate could also be made with H_2S or with phosphate solutions having a lower pH (2.5–6.5), but slightly more activity was recovered by the method described above.

The dry supernatant from the previous step was ground and shaken with 7.5 l. of absolute methanol, which was then decanted off and treated with 0.4 volume of diethyl ether per volume of methanol. The resulting low-potency precipitate was removed. Most of the remaining activity was precipitated by the further addition of ether up to 50% by volume. The vacuum-dried precipitate had a dry weight of 3.7 g.

Since the factor is only slightly soluble in methanol, it was not all extracted from the dried supernatant by the above procedure. In a separate experiment, under similar conditions, 3 successive extractions dissolved, respectively, 60%, 30%, and 10% of the activity. The first extract was most active on a dry-weight basis.

The rhizoctonia factor is insoluble in ether, petroleum ether, benzene, ethyl acetate, acetone, butanol, and absolute ethanol. It is soluble in water and 70% ethanol, and diffusible through a Cellophane membrane. It is stable to heat (100°C., 15 min., pH 2.5). The potency of the original culture[2] corresponded to about 6 g. of the concentrate/l. of culture, of which 10% was recovered.

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[2] This refers to total potency less potency due to the Aspergillus factor. The latter could be measured separately in the whole culture, because *A. niger* is not inhibited by the rhizoctonia factor.

Concentration of the Aspergillus Factor

The subtilin-free butanol extract (300 ml., representing 800 ml. original culture) was mixed with 900 ml. of petroleum ether (30°–60°). This was extracted twice with 700-ml. aliquots of pH 7 phosphate buffer (containing 0.25% KH_2PO_4). The combined extracts were extracted with ¼ volume of anhydrous butanol. The butanol was evaporated *in vacuo*, leaving 0.45 g. of viscous brown material (Fraction I).

During the first of the above extractions, about 15 ml. of emulsion formed between the butanol-petroleum ether and the aqueous layers. This was collected separately and evaporated to dryness, taken up in 150 ml. of water, brought to pH 7, and extracted with ¼ its volume of anhydrous butanol, which was then evaporated to dryness. The residue was taken up in water and lyophilized, giving 23 mg. of grayish white powder (Fraction II).

Both Fractions I and II are insoluble in ether but soluble in water, anhydrous ethanol, and anhydrous butanol. They are stable to heat (100°C., 15 min., pH 2.5), and will not diffuse through a Cellophane membrane. They may be extracted by butanol from water over a pH range of 0.5 to 11. Fraction II, however, loses some of its activity at a pH of 0.5 to 1.5. The fractions also have similar antibiotic spectra, but differ markedly in their potencies. Fraction II had 60 times the potency of Fraction I against *R. solani* and 70 times against *A. niger*. Fraction II thus appears more highly purified, and its potency against various organisms is shown in Table I. The approximate yields of these concentrates per liter of *B. subtilis* culture were 550 mg. for Fraction I and 30 mg. for Fraction II. These account for all of the aspergillus activity of the original culture.

Antibiotic Spectra

The rhizoctonia factor and the aspergillus factor have different spectra. Table I gives the concentration required for 50% inhibition of 12 fungi and of *Micrococcus Conglomeratus* and for 95% inhibition (estimated) of 7 yeasts.

A bacitracin concentrate was compared with the two concentrates of antifungal factors. Concentrations causing 50% inhibition of *R. solani* (in p.p.m.) were: bacitracin, 500; rhizoctonia factor, 50; aspergillus factor, 4. Corresponding figures for inhibition of *Micrococcus conglomeratus* were: bacitracin, 0.7; rhizoctonia factor, >180; aspergillus factor, >13. The high activity of bacitracin against *M. conglomeratus* differentiates it from the antifungal factors. Other known *B. subtilis* antibiotics have not been included in these studies.

Effect of Culture Method on Activity

Whole *B. subtilis* cultures grown in various ways were assayed against *R. solani* and *A. niger*. Their activity against these organisms varied greatly with the culture method and medium (Table II). The method finally adopted for large-scale subtilin production (6) was not the best for production of antifungal activity (last line, Table II).

TABLE II

Effect of Cultural Conditions on Antifungal Activity

Cultural conditions	Percentage of whole culture causing 50% inhibition when added to the assay medium	
	R. solani	*A. niger*
Asparagus juice medium, submerged culture	1.6	8
Beet molasses, surface culture	0.1–0.2	12
Asparagus juice, surface culture	2.3	8
10% Asparagus juice concentrate, 2.5% Steffens waste, submerged culture (6)	2.6	100 (approx.)
16% Asparagus juice concentrate, 5% Steffens waste, submerged culture	1.6	5
Semi-synthetic medium, submerged culture (6)	1.7	17

ACNKOWLEDGMENT

The *B. subtilis* cultures from which the antifungal factors were isolated were grown by R. E. Feeney and J. A. Garibaldi of this laboratory. The antibacterial assays were made by E. M. Humphreys of this laboratory. Cultures of test fungi were supplied by the Division of Plant Pathology, University of California, Berkeley; and the bacitracin concentrate by Bristol Laboratories, Inc., Syracuse, N. Y.

SUMMARY

Bacillus subtilis cultures, grown for subtilin production, also produce at least two other antibiotic substances. Unlike subtilin, they are active against fungi but relatively inactive against bacteria. They are similarly distinguished from bacitracin. One (rhizoctonia factor) is soluble in water, absolute methanol, and 70% ethanol, but insoluble in ether, petroleum ether, benzene, ethyl acetate, acetone, butanol, and absolute ethanol. It diffuses through a Cellophane membrane. The concentrate was active against 8 out of 11 fungi and one out of 7 yeasts at concentrations ranging between 6 and 600 p.p.m. The other (aspergillus factor) is insoluble in diethyl ether or petroleum ether but soluble in water, anhydrous butanol, and anhydrous ethanol. It will not diffuse

through a Cellophane membrane. The concentrate described was active against all of 11 test fungi and 6 out of 7 yeasts in concentrations between 3.5 and 85 p.p.m.

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Growth-Depressing Fractions in Raw Lima Beans

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INTRODUCTION

As a part of investigations on effects of processing methods on final biological values of legume proteins, this laboratory has been interested in the purification and characterization of the factor or factors in raw lima beans that inhibit trypsin *in vitro* and depress growth in the rat. Recently (1) we described the unexpected growth-depressing activity of this factor in casein hydrolyzate diets. The present report deals with biological characterization of the inhibiting substance, its partial purification, the magnitude of its effect, and methods of overcoming the effect.

The presence of a trypsin inhibitor in legumes was first reported by Ham and Sandstedt (2) and Bowman (3) in 1944. Ham, Sandstedt and Mussehl (4) later showed that an acetone-insoluble soybean fraction containing this factor inhibited growth of chicks. A similar growth-depressing effect of the pH 4.2-soluble, 2.7 *M* $(NH_4)_2SO_4$-insoluble fraction of soybean flakes was demonstrated for the rat by Klose, Hill and Fevold (5). Kunitz (6) isolated a trypsin inhibitor in crystalline form from soybean meal by a modification of the bentonite adsorption method of Alderton, Ward and Fevold (7), and determined its properties. Recently, Bowman (8) has indicated the presence of two trypsin inhibitors in soybeans: one insoluble in 60% alcohol and 40% saturated $(NH_4)_2SO_4$ and similar to Kunitz's crystalline material; the other soluble in these two solutions, insoluble in acetone, and soluble and stable in 2.5% trichloroacetic acid at 98°C. for 5 min. Quantitative

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in vitro determinations of the trypsin inhibitors present in an extensive list of leguminous seeds, including lima beans, have been reported by Borchers *et al.* (9).

EXPERIMENTAL

General Procedure

Determinations of growth inhibition were carried out with young (70–90 g. wt.) albino rats of the Sprague-Dawley strain. All diets contained 50% by weight of basal mixture, designed to furnish in adequate amounts all dietary essentials except protein, and containing (per cent): cottonseed oil, 5; U. S. P. cod liver oil, 2; 5.5% tocopherol concentrate, 0.3; brewers' yeast, 2; choline chloride, 0.1; salt mixture (McCollum's No. 185 plus trace elements), 4; corn starch, 18.3; sucrose, 18.3; and (mg.-%) thiamin chloride, 0.2; riboflavin, 0.5; pyridoxin, 0.2; calcium pantothenate, 2.5; and nicotinic acid, 1.0. The various protein supplements, including the lima bean fractions, were added at an established total crude protein level and the remainder of each diet made up of an equal weight mixture of corn starch and sucrose.

Previous studies had indicated that the average gains in weight over a 5 day period were completely reliable indices of relative gains in weight over 2 to 4 week periods. Weight curves demonstrating this point are shown in Fig. 1. Accordingly, to reduce the preparatory work for the production of the various protein fractions, a standard 5 day feeding period was used in most cases. Animals were standardized by selection of those gaining at a rate within definite limits during a preëxperimental period on a completely adequate diet Groups consisted of equal numbers of males and females, with 8–10 rats per group, and with each litter divided as equally as possible among all groups of the experiment. Records were kept of individual daily weights and average feed consumption for males and females. The results are expressed as feed consumed per rat per day, average gain or loss in weight per rat per day, and gain per g. of nitrogen (protein and amino acid) consumed. All values represent means of male and female averages, unless otherwise specified.

I. Growth Tests on Whole Lima Beans

Rats were fed diets containing 14% crude protein derived from a 66.7% level of ground whole baby lima beans. The basal mixture in this case was reduced to 33% of the complete diet by subtraction of 17% of starch-sucrose. Diets were fed to equivalent groups of 8 rats each for 13 days. The single and combined effects of steam heating and methionine supplementation were studied. The results are given in Fig. 1. The inability of either treatment alone to bring the lima beans up to the performance of the casein control and the adequacy of the combined treatments are at once apparent. It should be noted that the growth depression on the raw lima beans is a composite effect, representing such factors as growth-inhibiting substances, low biological value (amino acid deficiencies) of the lima bean protein, and palatability. The effects of the last two factors are reduced in varying degrees in the following experiments with protein fractions of the lima beans.

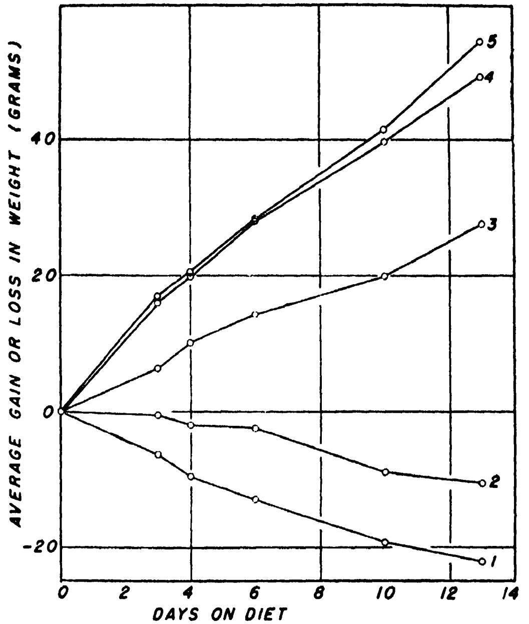

FIG. 1. The effect of steam heating and methionine supplementation of raw lima beans on their growth-promoting ability. 1. 66.7% ground raw lima beans. 2. 1 plus 0.6% methionine. 3. 1 steamed at 15 lb. press. for 15 min. 4. 1 steamed 15 lb. 15 min. plus 0.6% methionine. 5. Casein.

II. Growth Tests on Crude Protein Fractions Prepared from Lima Beans

Two batches of crude lima bean protein were prepared in the following manner: The ground beans were extracted with 2% NaCl solution, the extract was filtered, and the crude protein precipitated at 4 *M* $(NH_4)_2SO_4$ concentration, dialyzed salt free, and lyophilized (L. B. No. 1). This product contained 72% crude protein. The other preparation (L. B. No. 2) differed from L. B. No. 1 only in that extraction was accomplished with 0.25 *N* H_2SO_4 after first extracting the ground beans with 80% alcohol. It contained 75% crude protein. The two preparations were tested in parallel feeding experiments, with results as indicated in Table I. Preliminary heating tests on crude lima bean protein had indicated that a maximum beneficial effect on growth-promoting ability was obtained with a 15 min. treatment at 15 lb. steam pressure, or a 60 min. steaming at atmospheric pressure. The occurrence of a definite maximum effect as the heating time is increased is similar to results obtained with soybeans (10). A 15 min., 15 lb. steam pressure treatment was used in the experiments described here. From an inspection of Table I, we note a very slight improvement of the unheated protein by addition of methionine alone, a much greater effect by steam heat alone, and, with a combination of the two, an increase to an optimum rate of growth.

The quality of the basal dietary protein might be expected to have an effect on the amount of inhibition produced by a given amount of growth inhibitor. Casein and steam-heated, hexane-extracted soybean flakes were used as the base protein in a series of growth tests with the lima bean growth inhibitor. Hexane-extracted soybean flakes were heated at 15 lb. steam pressure for 15 min. to destroy all growth inhibitor present. Data in Table II indicate the similar results obtained with the two base proteins.

TABLE I

Effect of Steam Heating and Methionine Supplementation on Crude Lima Bean Protein
(15% total crude protein; 10 rats per group)

L. B. No. 1—Lima bean fraction, soluble in 2% NaCl at pH 7.4, insoluble in 4 *M* $(NH_4)_2SO_4$, containing 72% crude protein.

L. B. No. 2—Lima bean fraction, soluble in 0.25 *N* H_2SO_4, insoluble in 4*M* $(NH_4)_2SO_4$, containing 75% crude protein.

Group	Protein supplement (15% crude protein)	Feed consumption	Average 12 day gain or loss	Gain/g. of N consumed
		g./rat/day	*g./rat/day*	*g.*
1	21% L. B. No. 1	5.3	−2.2	—
2	21% L. B. No. 1+15 lb. steam, 15 min.	11.3	2.9	10.8
3	20.5% L. B. No. 1+0.5% methionine	5.4	−1.4	—
4	20.5% L. B. No. 1+15 lb. steam; 15 min.+0.5% methionine	10.8	4.7	18.2
5	19.5% L. B. No. 2	3.9	−2.6	—
6	19.5% L. B. No. 2+15 lb. steam, 15 min.	8.2	1.6	8.1
7	19.0% L. B. No. 2+0.5% methionine	4.6	−2.4	—
8	19.0% L. B. No. 2+15 lb. steam, 15 min.+0.5% methionine	10.0	4.6	19.2

The influence of addition of methionine on the growth inhibition effected by lima bean protein fractions in casein diets was investigated. In this experiment the total crude protein level was raised to 17.5% in order that the effect of adding methionine to the casein alone would not be excessive. Methionine at 0.6% markedly counteracted the growth-depressing effect of the lima bean fraction (Table III). The lower growth rate on the 1.2% level of methionine, compared to the 0.6% level, corresponds to the report of McKittrick (11) that excessive methionine depresses the growth of chicks.

TABLE II

Comparison of Casein and Heated Soybean Flakes as the Base Protein in Inhibition Tests

(15% total crude protein, 10 rats per group)

L. B. No. 3—Lima bean fraction, soluble in 2% NaCl, insoluble in 4 *M* $(NH_4)_2SO_4$.
L. B. No. 3 heated—L. B. No. 3 heated at 15 lb. steam press., 20 min.

Group	Per cent crude protein from			Feed consumption	Average 15 day gain	Gain/g. of N consumed
	Heated soybean	L. B. No. 3	L. B. No. 3 heated			
				g./rat/day	*g./rat/day*	*g.*
1	15	—	—	10.4	3.1	12.4
2	10	5	—	9.5	1.5	6.6
3	10	—	5	12.1	3.1	10.7
4	10	2.5	2.5	11.7	2.5	8.9
5	12.5	2.5	—	11.7	3.0	10.7
	Casein	L. B No. 3	L. B. No. 3 heated			
6	15	—	—	10.8	3.7	14.3
7	10	—	5	13.0	4.3	13.8
8	10	5	—	10.2	1.8	7.3

TABLE III

The Ability of Methionine to Counteract the Growth Inhibition Produced by Lima Bean Salt Fractions in Casein Diets

(17.5% total crude protein, 10 rats per group)

L. B. No. 4—Lima bean fraction, soluble in 0.4 *M* and insoluble in 2 *M* $(NH_4)_2SO_4$, soluble at pH 5.

Group	Nitrogen supplement in addition to casein[a]	Feed consumption	Average 5 day gain	Gain/g. N consumed
		g./rat/day	*g./rat/day*	*g.*
1	None	12.9	3.9	11.5
2	Methionine 0.6%	12.8	4.4	12.2
3	Methionine 1.2%	11.6	3.3	10.2
4	L. B. No. 4 4.6%	11.5	1.6	5.4
5	L. B. No. 4 4.6%+methionine 0.6%	11.9	3.4	10.5
6	L. B. No. 4 4.6%+methionine 1.2%	11.9	3.1	8.9

[a] Casein protein decreased by amount of lima bean protein present, methionine added in addition to 17.5% crude protein level.

III. Growth Tests on Concentrated Lima Bean Inhibitor Preparations

Lima beans were fractionated according to the method of Kunitz (6) for soybeans, with only slight modifications. Ground lima beans were extracted with 80% ethyl alcohol at room temperature and the extract was discarded. The residual meal was extracted with 0.25 *N* H_2SO_4, and the growth-inhibiting activity adsorbed from this extract on bentonite. Elution of activity from bentonite was accomplished with aqueous pyridine solution, and the pyridine removed by dialysis. The resulting solution was then precipitated with $(NH_4)_2SO_4$ and the precipitate finally dialyzed salt-free and dried by lyophilization. The results of the growth tests summarized in Table IV demonstrate the high growth-inhibiting activity of the adsorption preparation in comparison to crude protein fractions evaluated in Tables II and III. It can be estimated that the adsorption technique produced about a 10-fold concentration of activity over that of the crude, salt-soluble, $(NH_4)_2SO_4$-insoluble fraction. *In vitro* tests of the trypsin-inhibiting activity of these two fractions have shown about a 20-fold difference in activities, the adsorption preparation having the higher activity. Chemical characterization studies of the most highly purified lima bean preparation, which has as high an *in vitro* activity as the crystalline trypsin inhibitor from soybeans, will be published elsewhere (12). Comparative tests with purified trypsin-inhibiting fractions derived from hexane-extracted soybean flakes (Exp. B) illustrate the similar

TABLE IV

Growth-Depressing Effect of Most Highly Concentrated Growth Inhibitor from Lima Beans. Comparison with Crystalline Trypsin Inhibitor from Soybeans

L. B. No. 5—Lima bean fraction prepared by adsorption method, precipitated with 2 *M* $(NH_4)_2SO_4$.

S. B. No. 1—Trypsin inhibitor from hexane-extracted soybean flakes, prepared according to method of Kunitz (6).

Exp. A. 14.4% total crude protein; 10 rats/group

Group	Protein supplement in addition to casein[a]	Feed consumption	Average 5 day gain	Gain/g. of N consumed
		g./rat/day	*g./rat/day*	*g.*
1	None	10.6	2.5	10.3
2	L. B. No. 5—0.11%	10.5	2.3	9.6
3	L. B. No. 5—0.36%	9.0	1.2	5.8
4	L. B. No. 5—0.54%	8.9	0.8	3.9

Exp. B. 15.0% total crude protein; 10 rats/group

Group	Protein supplement in addition to casein[a]	Feed consumption	Average 5 day gain	Gain/g. of N consumed
1	None	12.5	3.4	11.3
2	S. B. No. 1—1.00%	10.3	0.6	2.4

[a] Casein protein decreased by amount of legume protein present.

behavior and relative activities of the lima bean and soybean preparations. Distribution of growth-inhibiting activity between various fractions of crude soybean protein has been reported previously (5).

Because of the variation between experiments with respect to age of rats and type and level of base protein used, it is important not to make direct comparisons between growth rates in two separate experiments in the work reported above. Rather, comparisons should be made between the relative (%) decreases in growth rate (or g. gain/g. food consumed) based on the value obtained with the positive control base protein in each experiment.

DISCUSSION

It is difficult to estimate the amount of purification of the growth inhibitor effected by chemical fractionation, starting from the raw lima bean. In the relatively large quantities of raw beans or crude protein fed we are introducing two factors contributing to poor growth: first, the effect of the growth inhibitor and second, the poor quality of the lima bean protein, which is apparent from the limited growth obtained with the heated (and hence inhibitor-inactivated) material. In the case of the purified, highly active lima bean fractions, the amount of lima bean protein introduced is too small to have any significant effect on overall protein quality. Hence, we may estimate that a 10-fold concentration of the active principle is accomplished in going from a crude, salt-soluble $(NH_4)_2SO_4$-precipitable fraction to a preparation carried through adsorption and elution with bentonite.

The mode of action of the growth inhibitor is yet to be completely explained. It has been generally accepted that the beneficial effect of steam heat on raw beans is due to the increased availablility of certain amino acids present in the protein. The remarkably high concentration of trypsin-inhibiting activity in the growth-inhibiting fractions of legumes suggests that the effect is one of decreased availability due to impaired digestion. However, several observations cast doubt on the assumption that inhibition of enzymatic digestion is the principal or only factor involved. Sulfur- and nitrogen-balance experiments run on rats fed raw and heated soybeans, as reported by Johnson, Parsons and Steenbock (13), showed that heating did not increase absorption of the sulfur-containing amino acids but did increase the utilization of these amino acids after absorption. Two reports have appeared in which the inhibiting fraction of soybeans (14) and of lima beans (1) were shown to be active in hydrolyzed protein diets where the inhibition of normal enzymatic hydrolysis would not be expected to be a factor. Melnick *et al.* (15) have attributed the poor biological value of raw soybean protein to impaired rate, rather than extent, of enzymic liberation of methionine compared to the other essential amino acids.

Methionine supplementation of the unheated lima bean fractions gave only a small improvement, pointing to the possibility of a multiple deficiency with respect to available amino acids. Steam heating of the raw beans, or the crude extracted fractions, resulted in a marked improvement. The optimum times of heating correspond roughly to those found for soybeans (10). However, the steamed fractions were still considerably below the performance of the casein control; the two gave comparable growth only after supplementation of the heated beans with methionine. This behavior corresponds to the known methionine deficiency of lima bean protein calculated from amino acid analyses.

SUMMARY

Lima beans and various fractions derived from them were shown to be good sources of a growth inhibitor for rats. The inhibitor was concentrated in the crude protein fraction, from which it could be purified about 10-fold by the Kunitz bentonite adsorption procedure. Gross solubility and stability characteristics, and the trypsin-inhibiting activity of the factor or factors were found to be similar to those of the soybean growth inhibitor. Growth, either on the raw beans or on their crude protein, was slightly improved by addition of methionine, markedly increased by steam heating, and made approximately equal to growth on a casein control diet by a combination of the two. Growth depression by purified lima bean fractions was demonstrated in diets containing either casein or heated soybean as the base protein. Methionine almost completely counteracted the growth inhibition produced by highly purified fractions of the inhibitor in casein diets.

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The Enzyme-Substrate Compounds of Horseradish Peroxidase and Peroxides. II. Kinetics of Formation and Decomposition of the Primary and Secondary Complexes *

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In previous studies of the kinetics of the reaction of peroxidase and H_2O_2 (1), it was found that enzyme and substrate combine very rapidly ($k_1 = 1.2 \times 10^7\,M^{-1} \times \text{sec}^{-1}$). But it was difficult to demonstrate that this reaction was of the second order over a wide range of H_2O_2 concentrations since that apparatus was equipped only with color filters and did not distinguish between the kinetics of formation of the primary and secondary complexes. An improved apparatus incorporating a monochromator has been developed (2), and the kinetics of the primary complex are measured without interference from the secondary complex and are shown to be a true second-order reaction up to the time limit measurable with this apparatus ($\sim$ 3 millisec.).

Now it is possible to follow the transition of the primary to the secondary complex and to show that this is a first-order reaction in the absence of acceptors. It is also found that the primary complex is converted into the secondary complex before the enzyme is liberated from the enzyme-substrate complex—in contrast to the catalase-peroxide reactions (3). In addition, some of the factors affecting the transition from the primary to the secondary complex have been investigated.

The kinetics of formation of the primary and secondary complexes of peroxidase and methyl or ethyl hydrogen peroxide have also been measured.

The lack of an appreciable pH effect upon the dissociation constant of the peroxidase-peroxide complexes indicates that the heme-linked hydroxyl group found by Theorell (4) does not interfere with the formation of the enzyme-substrate complexes and that peroxidase combines with the peroxide group as shown below.

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* This is No. VIII of a series of papers on catalases and peroxidases.

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The reactions studied in this paper are those of the formation of Complex I,

$$\text{Per}\cdot\text{OH} + \text{HOOR} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{Per}\cdot\text{OOR} - \text{I} + H_2O, \tag{1}$$

its transition into Complex II,

$$\text{Per}\cdot\text{OOR} - \text{I} \xrightarrow{k_7} \text{Per}\cdot\text{OOR} - \text{II}, \tag{2}$$

and the decomposition of Complex II in the presence of an acceptor, AH_2,

$$\text{Per}\cdot\text{OOR} - \text{II} + AH_2 \xrightarrow{k_4 = \frac{k_3}{AH_2}} \text{Per}\cdot\text{OH} + \text{ROH} + \text{A}. \tag{3}$$

The Kinetics of Formation and Decomposition of the Primary and Secondary Compounds with H_2O_2

The kinetics of Fig. 1 were obtained with the rapid-flow apparatus equipped with a monochromator (2) and verify the spectral data of Fig. 3 in (5). In the oscillograph records the top trace gives the flow velocity indication—the base-line means that the flow velocity is zero; the liquid is stopped in the capillary. A downward deflection indicates that the fluid is moving from the mixing chamber to the point of observation at a velocity corresponding to a time after mixing of $\left[\frac{28}{\text{deflection in mm.}}\right]$ millisec. Before the flow starts, the material in the observation tube is that remaining from the previous experiment and is usually free peroxidase (providing time is permitted for the previously-formed peroxidase complexes to decompose completely). In any case, the optical density during the flow of peroxidase and peroxide through the capillary under the conditions of Fig. 1 represents the optical density of free peroxidase. This is also independently controlled by filling the observation tube with a mixture of enzyme and buffer, peroxide being omitted. The H_2O_2 and peroxidase were so dilute in this experiment that the formation of Complex I did not begin until the flow velocity had nearly stopped; therefore, the reaction kinetics are measured directly by means of the time scale on the records.

In Fig. 1 at 427 mμ is seen the relatively slow formation of Complex II as would be expected since the data of Fig. 3 of (5) show an isosbestic point between peroxidase and Complex I at this wavelength; therefore, the kinetics of the formation of Complex II only are measured at this wavelength. At 395 mμ, the more rapid formation of

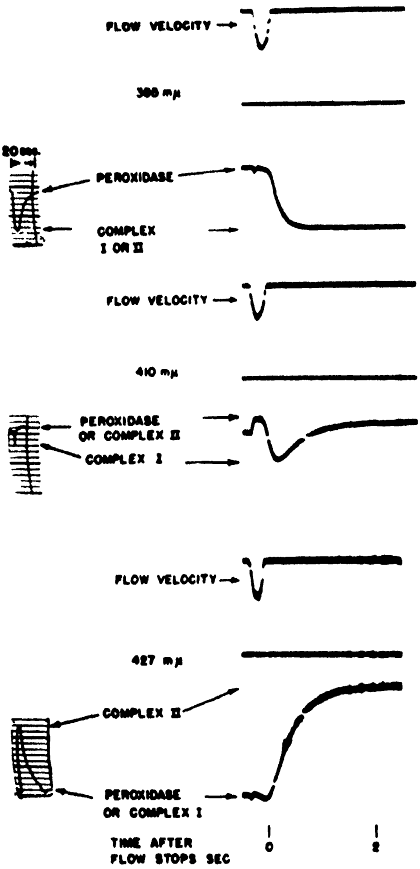

FIG. 1. The kinetics of formation and decomposition of the primary and secondary compounds of horseradish peroxidase and H_2O_2. The 3 wavelengths selected are isosbestic points for Complexes I and II at 395 mμ, for free peroxidase and Complex II at 410 mμ, and for free peroxidase and complex I at 427 mμ. The left-hand traces show both the kinetics of formation and disappearance of the complexes, and the right-hand traces show only the initial phases of the reactions. 0.98 μM horseradish peroxidase, 1.7 μM H_2O_2, 0.001 M phosphate buffer, pH = 7.0 (Exp. 301).

Complex I is shown; however, the trace remains quite steady while compound II is forming at 427 mμ since compounds I and II are isosbestic at 395 mμ in accord with Fig. 3 of (5). As the left-hand records on a slow time scale indicate, the complexes at both 427 and 395 mμ slowly decompose into free peroxidase. At 411 mμ, the complete kinetics of Complex I are seen; the compound rapidly forms and then disappears as it is converted into Complex II. No further changes occur as the left-hand record on the slow time scale shows. Since the final density is the same as that of free peroxidase, this wavelength is an isosbestic point for peroxidase and compound II. The relations of these 3 curves are quantitatively analyzed in Fig. 5.

Fig. 3 of (5) indicates that the kinetics of formation of the primary complex can be measured at the region 370–395 or at 411 mμ.[2] Kinetic studies of the formation of compound I are made near 390 mμ because the calibration of the optical density of the free enzyme is more readily obtained in the region 370–395 mμ after compound II has decomposed, and the wavelength setting is not critical in this spectral region. The wavelength 427 mμ is satisfactory for studying the kinetics of compound II since Complex I and peroxidase are isosbestic, and therefore the wavelength setting is not critical.

TABLE I

The Kinetics of Formation of the Primary and Secondary Complexes of Horseradish Peroxidase and H_2O_2

1.3 μ*M* horseradish peroxidase, pH = 4.7, 0.01 *M* acetate. (Exp. 163d)

The various formulae used to compute *k* are appropriate to the various initial peroxide concentrations.

Formation of primary complex at 390 mμ	Hydrogen peroxide concentration—μ*M*	0.5	1.0	2.0	4.0	20
	Oscillograph deflection (D_1) at 390 mμ—mm.	40	60	58	(59)	(59)
	Oscillograph deflection (D_2) at 390 mμ during flow—mm.	—	—	—	50	22
	Flow velocity indication (f)—mm.	—	—	—	19	17
	Half-time for formation of complex ($t_{1/2}$)—sec.	0.08	0.10	(0.06)	—	—
	$k = \frac{0.46}{t_{1/2}}$, $k = \frac{1}{t_{1/2}}$, and $k = 27f \log \frac{D_1}{D_2}$ sec^{-1}	5.7	9	—	36	190
	$k_1 = \frac{k}{[H_2O_2]}$ M^{-1} sec^{-1} $\times 10^{-7}$	7	9	—	9	10
Formation of secondary complex at 430 mμ	Oscillograph deflection at 430 mμ—mm.	53	72	75	73	78
	Half-time for formation of complex—sec.	0.18	0.20	0.20	0.30	0.12
	$k_7 = \frac{0.7}{t_{1/2}}$—sec^{-1}	3.8	3.5	3.5	2.3	5.7
Decomposition of secondary complex at 430 mμ	Half-time for decomposition of complex—sec.	27	53	—	—	—
	$k_3 = \frac{x_0}{p_{max}\, t_{1/2}\ \text{off}}$ sec^{-1}	0.017	0.023	—	—	—

Table I summarizes kinetic data on the primary and secondary complexes. Up to a peroxide concentration of 2.0 μ*M*, the "stopped flow" method is suitable; neither method is suitable between 2 and 3 μ*M*, and between 4 μ*M* and 20 μ*M*, the flow method is suitable for measuring the reaction kinetics. It is seen that the value of k_1 is nearly constant as is required for a bimolecular reaction of enzyme and substrate. Also the mean value ($0.9 \pm 0.2 \times 10^7\ M^{-1} \times \text{sec}^{-1}$) is in accord with that obtained previously with partially purified peroxidase ($1.2 \pm 0.4 \times 10^7\ M^{-1} \times \text{sec}^{-1}$) (1).

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[2] In each experiment the isosbestic point is found experimentally, and the wavelength is read from the monochromator scale. As will be apparent from the values given in this paper, the isosbestic point was found to be between 410 and 411 mμ. The differences are mainly caused by the coarse graduations on the monochromator dial and by the rather large spectral interval, 7.5 mμ.

The great difference in the effect of changes in initial peroxide concentration upon the kinetics of formation of Complex I and Complex II is clearly shown in Fig. 2. Whereas the rate of formation of the primary complexes increases linearly with the peroxide concentration, the rate of formation of the secondary complexes does not. However, the curves for k_1 [peroxide] and for k_7 intersect at small values of peroxide concentration. Below this value of peroxide concentration, k_7 will vary with peroxide concentration in the same way as will k_1 [peroxide]. With the peroxidase preparation used here, these curves intersected at $k_7 = 4\ \text{sec}^{-1}$. But with a less pure preparation used in earlier experiments (1), this intersection apparently occurred at $k_7 = 35\ \text{sec}^{-1}$. In that case, accurate values of k_1 were obtained by measuring the variation of k_7 with H_2O_2 concentrations less than about 10 μM.

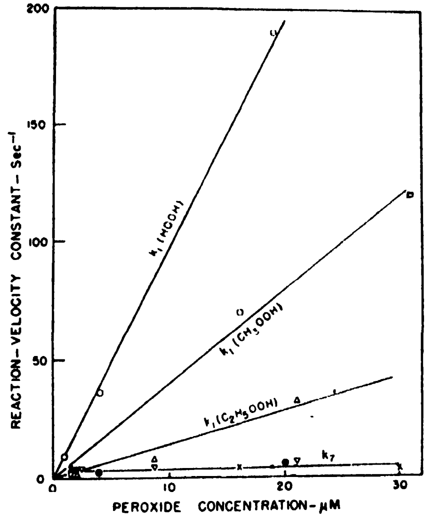

FIG. 2. The effect of peroxide concentration upon the rate of formation of the primary complexes ($k_1 \times$ [peroxide]) measured at 390 mμ and the rate of formation of the secondary complexes (k_7) measured at 427 mμ. Note the intersection of the curves for k_1 with that for k_7 near the origin. Data are selected from Tables I, II, and III. The symbols ●, ∇, and x represent values of k_7 for H_2O_2, methyl hydrogen peroxide, and ethyl hydrogen peroxide, respectively.

Above the intersection of the curves for k_7 and $k_1[H_2O_2]$, the rate of formation of the secondary complex (k_7) does not increase appreciably with the H_2O_2 concentration, and it is concluded that this compound is formed from the primary complex in a first-order reaction of $k_7 = 4.0$ sec^{-1}.

Under these experimental conditions, the velocity constant for the decomposition of the secondary complex is about 0.02 sec^{-1} as calculated from the usual formula (1).

Here, as before (1), an accurate measurement of the dissociation constant of the peroxidase-hydrogen peroxide complex by direct titration is difficult; the usable enzyme concentrations are about 100 times greater than the probable value of the dissociation constant. Kinetic data give 2×10^{-9} M (k_3/k_1) but this assumes that $k_2 = 0$. More accurate titration data are given below.

Methyl Hydrogen Peroxide

The sequence of records in Fig. 3 demonstrates kinetically the existence of primary and secondary complexes of peroxidase and methyl hydrogen peroxide. In fact, the data are identical to those obtained with H_2O_2 except that the events take place on a slower time scale—both compounds appear at 390 mμ, only compound I at 411 mμ and only compound II at 427 mμ. One difference between these records and those of Fig. 1 is that the capillary observation tube is filled with the secondary compound instead of free peroxidase before the initiation of the flow. After the initiation of the flow, the optical density of free peroxidase is obtained at the values of flow velocity used. As the flow stops, the formation of the complexes proceeds as described for Fig. 1.

Table II shows that peroxidase and methyl hydrogen peroxide combine in a bimolecular reaction to form the primary complex. The reaction velocity is, however, only about one-seventh as fast as with H_2O_2—the reaction velocity constant (k_1) being 1.5×10^6 $M^{-1} \times sec^{-1}$. Fig. 4 illustrates the increase of the speed of the formation of Complex I with an increase of methyl hydrogen peroxide concentration, with 4 μM methyl hydrogen peroxide, the reaction does not begin until the flow stops; with 100 μM, the reaction is half complete at the most rapid flow velocity.

The rate of formation of the secondary complex (k_7) is again found to be nearly independent of the peroxide concentration, and this complex

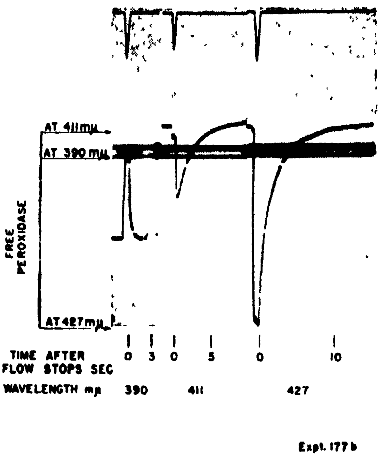

FIG. 3. The kinetics of formation and decomposition of the primary and secondary complexes of peroxidase and methyl hydrogen peroxide. These records are analogous to the right-hand traces of Fig. 1, with the exception that the observation tube is initially filled with Complex II instead of free peroxidase. 2.6 μM horseradish peroxidase, 4 μM methyl hydrogen peroxide, 0.01 M phosphate buffer, pH = 6.5 (Exp. 177). The rapid portions of these curves have been retouched.

TABLE II

The Kinetics of Formation of the Primary and Secondary Compounds of Horseradish Peroxidase and Methyl Hydrogen Peroxide

2.6 μM horseradish peroxidase, pH = 4.7, 0.01 M acetate (Exp. 173 a and b)

The various formulae used to compute k are appropriate to the various initial peroxide concentrations.

Formation of primary complex at 390 mμ	Methyl hydrogen peroxide concentration—μM	1.1	2.2	4.4	11	22	110
	Oscillograph deflection (D_1) at 390 mμ—mm.	11	31	41	42	(42)	(42)
	Oscillograph deflection (D_2) at 390 mμ during flow—mm.	11	31	41	42	38	25
	Flow velocity indication (f)—mm.	—	—	—	28	28	28
	Half-time for formation of complex—sec.	0.20	0.28	0.20	0.08	—	—
	$k = \frac{0.46}{t_{\frac{1}{2}}}$, $k = \frac{1}{t_{\frac{1}{2}}}$, $k = \frac{0.41}{t_{\frac{1}{2}}}$, $k = 27f \log \frac{D_1}{D_2}$ sec^{-1}	2.3	3.6	2.1	8.7	32	170
	$k_1 = \frac{k}{[CH_3OOH]}$ M^{-1}sec^{-1} $\times 10^{-6}$	1.4	1.5	1.0	1.0	1.5	1.5
Formation of secondary complex at 427 mμ	Oscillograph deflection at 427 mμ—mm.	—	—	90	87	89	89
	Half-time for formation of complex—sec.	—	—	0.22	0.18	0.12	0.13
	$k_7 = 0.7/t_{\frac{1}{2}}$ sec^{-1}	—	—	3.3	3.9	5.8	5.4
Decomposition of secondary complex at 427 mμ	Half-time for decomposition of complex—sec.	6	19	37	—	—	—
	$k_3 = \frac{x_0}{p_{max}\, t_{\frac{1}{2}} \text{ off}}$ sec^{-1}	0.30	0.06	0.05	—	—	—

is, therefore, considered to be formed from the primary compound in a first-order reaction. This secondary compound decomposes somewhat more rapidly than does that with H_2O_2, $k_3 = 0.06\ \text{sec}^{-1}$. The dissociation constant calculated from the ratio $\frac{k_3}{k_1}$ is $5 \times 10^{-8}\ M$ (for 2.2 and 4.4 μM methyl hydrogen peroxide) indicates the high enzyme-substrate affinity. The dissociation constant is determined from titration data later.

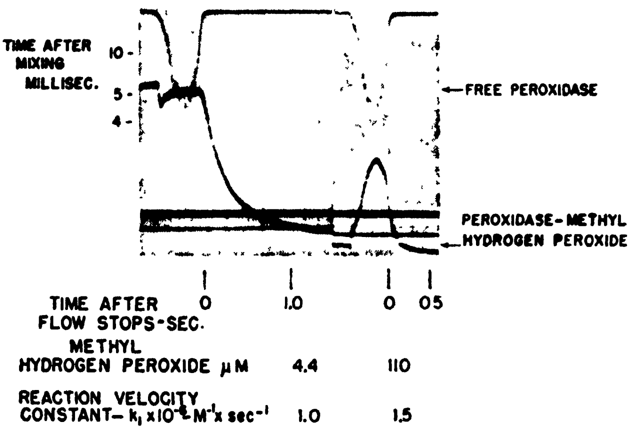

FIG. 4. Illustrating the second-order combination of peroxidase with methyl hydrogen peroxide. With dilute peroxide, the optical density corresponding to the free enzyme is obtained during the flow, and the reaction does not proceed appreciably until after the flow has stopped. The kinetics are here measured directly as a function of time, and $k_1 = 1 \times 10^6\ M^{-1} \times \text{sec}^{-1}$ (see Table II). With the stronger methyl hydrogen peroxide, the complex is about half-formed at the most rapid value of flow velocity, which, in this case, corresponds to a time after mixing of 3.5 millisec, and $k_1 = 1.5 \times 10^{-6}\ M^{-1} \times \text{sec}^{-1}$ (see Table II). 2.6 μM horseradish peroxidase, pH = 4.7 0.01 M acetate, $\lambda = 390\ \text{m}\mu$ (Exp. 173a).

Ethyl Hydrogen Peroxide

Table III shows that this primary complex is formed in a bimolecular reaction with a velocity constant which is somewhat larger than that obtained with methyl hydrogen peroxide, the mean value of k_1 being $3.6 \times 10^{-6}\ M^{-1} \times \text{sec}^{-1}$. The formation of the secondary compound is again a first-order reaction. The secondary complex decomposes at about the same rate as the H_2O_2 complex, and the dissociation constant of the enzyme-substrate complex, calculated from the ratio k_3/k_1, is about $5 \times 10^{-8}\ M$.

TABLE III

The Kinetics of Formation of the Primary and Secondary Compounds of Horseradish Peroxidase and Ethyl Hydrogen Peroxide

2.6 μM horseradish peroxidase, pH = 4.7, 0.01 M acetate buffer (Exp. 173 a and b)

The various formulae used to compute k are appropriate to the various initial peroxide concentrations.

	Ethyl hydrogen peroxide—μM	0.8	1.6	3.1	16	31	160
Formation of primary complex at 390 mμ	Oscillograph deflection (D_1) at 390 mμ—mm.	15	38	40	41	42	42
	Oscillograph deflection (D_2) at 390 mμ during flow—mm.	15	38	40	33	29	10
	Flow velocity indication—mm.	—	—	—	27	27	28
	Half-time for complex formation—sec.	0.08	0.12	0.10	—	—	—
	$k = \frac{0.5}{t_{\frac{1}{2}}}, \frac{0.27}{t_{\frac{1}{2}}}, \frac{0.21}{t_{\frac{1}{2}}}, 27f \log \frac{D_1}{D_2}$ sec^{-1}	6.3	2.2	2.1	70	120	470
	$k_1 = \frac{k}{[C_2H_5OOH]} M^{-1}$ sec$^{-1} \times 10^{-6}$	4.1	3.2	2.7	4.2	3.9	2.9
Formation of secondary complex at 427 mμ	Oscillograph deflection at 427 mμ—mm	—	—	87	85	90	88
	Half-time for complex formation—sec	—	—	0.22	0.16	0.18	0.13
	$k_7 = 0.7/t_{\frac{1}{2}}$—sec^{-1}	—	—	3.2	4.3	3.8	5.4
Decomposition of secondary complex at 427 mμ	Half-time for decomposition of complex—sec.	2.9	48	70	—	—	—
	$k_3 = \frac{x_0}{p_{\max}\, t_{\frac{1}{2}} \text{ off}}$ sec^{-1}	0.30	0.014	0.017	—	—	—

The Transition from Complex I to Complex II

Contrary to the experiments with catalase and peroxides, where the green primary compounds can decompose into free catalase with negligible formation of the secondary complex (6), these primary peroxidase-peroxide complexes have never yet been observed to decompose into the free enzyme except *via* the secondary complex. Figs. 5 and 6 are plotted from Figs. 1 and 3 and clearly show the mechanism of the transition from Complex I to Complex II. At 411 mμ, the concentration of Complex I rapidly rises to a maximum value and, at the same time, Complex I begins to be converted into Complex II, as is shown by the rise in the concentration of Complex II at 427 mμ after a slight induction period.

A possible mechanism is that the rate of formation of compound II is proportional to the concentration of compound I according to Eq. 2 above:

$$d[\text{Per(OOR)} - \text{II}] = k_7\,[\text{Per(OOR)} - \text{I}]\,dt, \tag{4}$$

$$\text{Per(OOR)} - \text{II} = k_7 \int_0^t \text{Per(OOR)} - \text{I}\,dt. \tag{5}$$

The equations show that the integral or area under the curve at 411 mμ should give the curve at 427 mμ. And in both Figs. 5 and 6 this is proved to be the case as the triangles (computed by graphical integration of the curve at 411 mμ) fall very nicely along the curve at 427 mμ. Eq. 5 is therefore verified. It appears, however, that the induction period at 427 mμ, especially in the case of H_2O_2, is longer than the simple theory would predict.

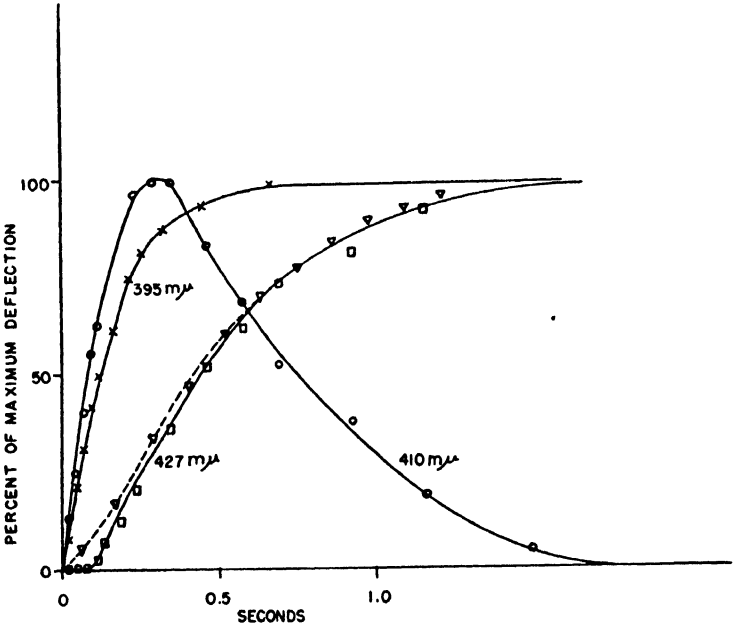

FIG. 5. The mechanism of the transition from the primary to the secondary complex of peroxidase and H_2O_2. The kinetic data of the 3 right-hand traces of Fig. 1 are plotted to the same scale. The triangles represent the integral of the kinetic curve of 410 mμ.

It is possible to evaluate k_7 in Figs. 5 and 6 since the scale factors are known. From Fig. 5, k_7 is 1.5 sec^{-1} from the integral of the curve at 411 mμ and 1.6 sec^{-1} from the measured half-time at 427 mμ. In Fig. 6, values of 0.5 and 0.7 sec^{-1} are obtained at 411 and 427 mμ, respectively. These values of k_7 are much larger than k_3 and ($\sim$ 0.03 sec^{-1}) and no correction for k_3 to the data of Figs. 5 and 6 are required.

At 390 and 395 mμ, where the spectra of Complexes I and II are isosbestic, there is seen to be no measurable change in the concentration of the sum of Complex I plus II. Therefore, the conversion from Complex I to Complex II is quantitative at this speed of transition. Even at considerably slower transition speeds (up to ~ 10 sec. for half-completion), no loss of Complex I in the formation of Complex II has been measured by this method (see Fig. 9). Thus, the stoichiometry represented by Eq. 2 is verified.

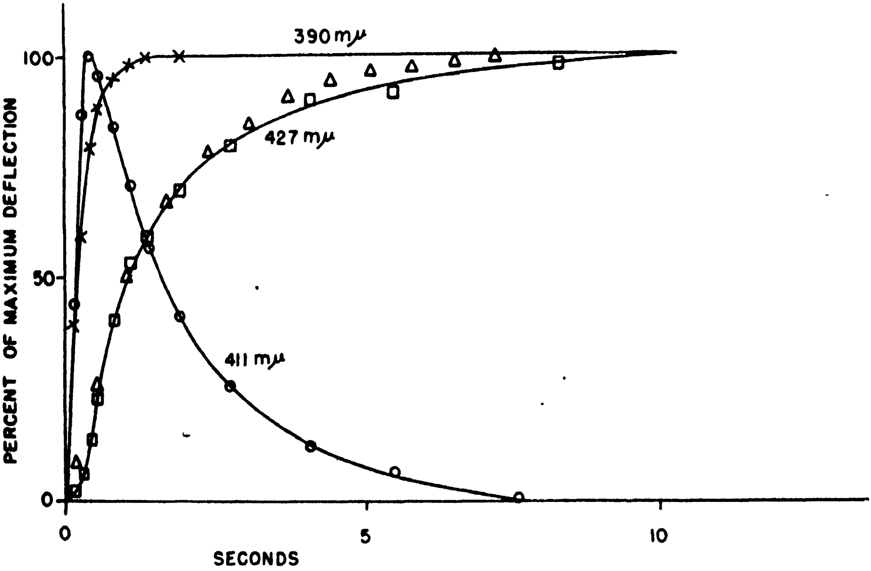

FIG. 6. The mechanism of the transition from the primary to the secondary complex of peroxidase and methyl hydrogen peroxide. The kinetic curves of Fig. 2 are plotted to the same scale. The triangles represent the integral of the kinetic curve at 411 mμ.

If the reaction of Eq. 2 were reversible, and mixtures of Complexes I and II were obtained under these experimental conditions, the isosbestic point at 411 mμ would change with peroxide and acceptor concentration—which does not occur. With very strong peroxidase (~ 200 μM), mixtures of Complexes I and II have been observed by visual spectroscopy (7). In dilute enzyme solutions, however, Eq. 2 does not appear to be reversible.

The Dissociation Constant of the Primary Complex.

The reaction kinetics of the primary and secondary complexes may be represented by the following equations which are a mathematical representation of Eqs. 1, 2, and 3. Let $p_1 = [\text{Per}\cdot\text{OOR} - \text{I}]$, $p_2 = [\text{Per}\cdot\text{OOR} - \text{II}]$, and $e =$ initial $[\text{Per}\cdot\text{OH}]$, $x = [\text{HOOR}]$ and $a = [\text{AH}_2]$. Then,

$$dp_1/dt = k_1x\,(e - p_1 - p_2) - (k_2 + k_7)\,p_1, \tag{6}$$

$$dp_2/dt = k_7p_1 - k_4ap_2. \tag{7}$$

In the steady-state defined by $dp_1/dt = 0$, Eq. 6 can be solved for p_1:

$$p_1 = \frac{x(e - p_2)}{x + Km_1}, \tag{8}$$

where

$$Km_1 = \frac{k_2 + k_7}{k_1}. \tag{9}$$

The quantity p_1 is a maximum when $dp_1/dt = 0$ and, at that time, Figs. 1 and 2 (and 5 and 6) show that p_2 is practically zero. Thus

$$p_1 = \frac{ex}{x + Km_1}, \text{ or } \left(\frac{e - p}{p}\right)x = Km_1. \tag{10}$$

In the steady-state defined by both dp_1/dt and $dp_2/dt = 0$, Eq. 7 can be solved for p_2 and on substituting for p_1 according to Eq. 8,

$$p_2 = \frac{k_7}{k_4a}\,\frac{x(e - p_2)}{x + Km_1} = \frac{xe}{\left(\frac{k_4a}{k_7} + 1\right)x + \frac{k_4a}{k_7}Km_1}. \tag{11}$$

In the absence of added acceptor, k_4a becomes equal to the spontaneous rate, k_3, which is about 0.1 sec^{-1}. Substituting $k_4a = k_3$ and $Km_1 = \frac{k_2 + k_7}{k_1}$,

$$p_2 = \frac{xe}{\left(\frac{k_3}{k_7} + 1\right)x + \frac{k_3}{k_1}\left(\frac{k_2}{k_7} + 1\right)}. \tag{12}$$

In the absence of acceptor, k_7 is about 4 sec^{-1} and $k_3/k_7 \ll 1$. Assuming $k_2/k_7 \ll 1$,

$$p_2 = \frac{xe}{x + Km_2}, \tag{13}$$

where

$$Km_2 = \frac{k_3}{k_1}. \tag{14}$$

This is the formula for K_m previously derived for peroxidase when $k_2 = 0$ (1).

From this analysis we would expect Km_1/Km_2 to be roughly equal to k_7/k_3; the dissociation constant of Complex I would be considerably larger than that of Complex II. Fig. 7 shows the results of titrations of peroxidase with methyl hydrogen peroxide in the rapid-flow apparatus. Methyl hydrogen peroxide was chosen because it combines comparatively slowly with peroxidase and, therefore, results in larger and more easily measurable dissociation constants than would be obtained with H_2O_2. The peroxidase solution used was as dilute as could be used in the rapid-flow apparatus with reliable readings at 411 mμ. The values of p_1 and p_2 are given by the maximum oscillograph deflection caused by mixing peroxidase with methyl hydrogen peroxide at 411 and 427 mμ, respectively, and these values are plotted in the left-hand portion of Fig. 7. The curves clearly show that Complex II has a higher affinity for peroxide than has Complex I. The dissociation constants are evaluated by plotting the data in the logarithmic form of Eq. 10, and $Km_1 = 3.2 \times 10^{-6}$ M and $Km_2 = 3.0 \times 10^{-7}$ M. As expected, Km_1 is larger than Km_2. Under these experimental conditions, $k_1 = 1.5 \times 10^{-6}$ $M^{-1} \times \text{sec}^{-1}$, $k_7 = 2.6$ sec^{-1} and $k_3 = 0.19$ sec^{-1}. If k_2 were equal to zero, $Km_1 = 1.7 \times 10^{-6}$ M, and $Km_2 = 1.3 \times 10^{-7}$ M from Eqs. 9 and 14. The values of k_2, which give the experimental values of Km_1 and Km_2 can be calculated directly from Eq. 9, $k_2 = 2.2$ sec^{-1}, and from Eq. 13 because $k_3/k_7 \ll 1$. and $Km_2 = \frac{k_3}{k_1}\left(\frac{k_2}{k_7} + 1\right) = 3.4$ sec^{-1}. Although no independent test of the value of k_2 has been made, the general agreement is highly satisfactory in view of the possible experimental error, and suggests that Eqs. 1, 2, and 3 correctly represent peroxidase kinetics under these experimental conditions.

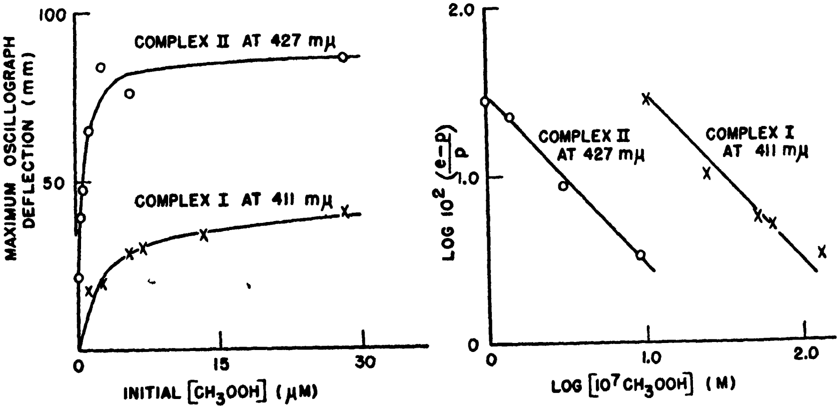

FIG. 7. Illustrating the difference between the dissociation constants of Complex I and Complex II. On the left, the deflections corresponding to the maximum concentration of these two complexes at 427 and 411 mμ are plotted against the initial methyl hydrogen peroxide concentrations. On the right, the data are plotted according to the logarithmic form of the mass-action equation, $\log\left(\frac{e-p}{p}\right) + \log\,[CH_3OOH] = \log K_m$, where e = initial peroxidase concentration (0.7 μM) and p is the maximum concentration recorded. The straight lines are drawn with 45° slopes and correspond to dissociation constants of 3.0×10^{-7} M for Complex II and 3.2×10^{-6} M for Complex I. Rapid-flow apparatus, 0.1 M acetate buffer, pH = 4.7 (Exp. 341).

Some Factors Decelerating the Transition from the Primary to the Secondary Complex

The speed at which this transition occurs apparently depends to a certain extent upon the purity of the peroxidase preparation. For example, Keilin and Mann (8) did not report this primary complex in their researches on peroxidase. In a previous paper on peroxidase, it is estimated that the primary complex shifted into the secondary one in a half-time of roughly 0.02 sec; so there was no possibility of detecting the primary complex by visual spectroscopy (1). Theorell's crystalline preparation apparently had a fairly slow transition, for he first saw the primary complex on addition of H_2O_2 to this preparation (9).

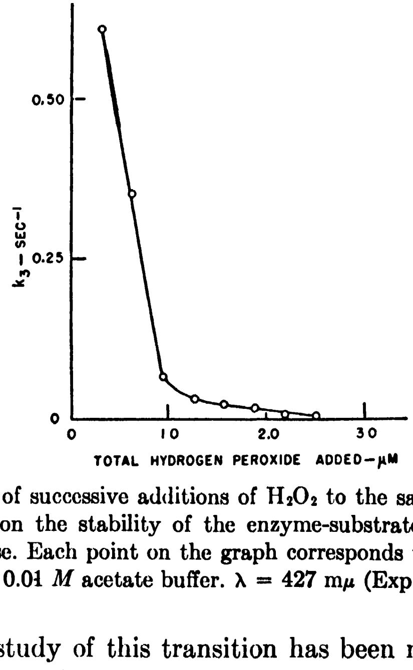

FIG. 8. The effect of successive additions of H_2O_2 to the same solution of horseradish peroxidase upon the stability of the enzyme-substrate complex. 0.104 μM horseradish peroxidase. Each point on the graph corresponds to the addition of 0.3 μM H_2O_2. pH = 4.7, 0.01 M acetate buffer. λ = 427 mμ (Exp. 167).

A preliminary study of this transition has been made. It has been found that the value of k_3, the velocity constant for the breakdown of the secondary complex, is a measure of the rate of the primary to secondary transition and is easier to measure (see Table V given later). As shown in Fig. 8, the treatment of the peroxidase solution with repeated additions of peroxide causes a marked decrease of k_3, from 0.62 to 0.01 sec^{-1}—a factor of 62. Such a peroxidase solution is said to be "stabilized." However, the first few additions of peroxide have by far the greatest effect as if some "acceptor" substance were present in either the enzyme preparation or the buffer solution and reacted with Complex II according to Eq. 3. Such an "acceptor" would have a concentration roughly equal to that of the enzyme and would be used up by these successive additions of peroxide. (If such a substance were an ordinary acceptor, its velocity constant, k_4, would be about the same as that of pyrogallol.) This experiment explains the initial high values of k_3 in Tables II and III. This experiment also suggests that it may not be the purity of the enzyme which determines the speed of transition from the primary to the secondary complex; it may be a question of the experimental conditions under which the test is made.

TABLE IV

The Effect of Acetate Buffer upon the Stability of Peroxidase-H_2O_2 Complex II

1.4 μM horseradish peroxidase (treated with several additions of H_2O_2)

0.86 μM H_2O_2, $\lambda = 425$ mμ, (Exp. 262a).

Buffer	Phosphate	Acetate	Acetate
Molarity—mM	0.5	5.0	50
pH	4.0	4.7	4.7
Recorder deflection ($p = e = 24$)	20	16	7
Half-time for decomposition of the complex—sec.	11	4.0	0.60
$k_3 = \dfrac{x_0}{p_{\max}\, t_{\frac{1}{2}} \text{ off}}$ sec^{-1}	0.07	0.24	3.6

In Table IV, the stability of Complex II in phosphate and acetate buffers is compared. With a previously "stabilized" peroxidase solution, a value of $k_3 = 0.07$ sec^{-1} is obtained in phosphate buffer while k_3 increases to 0.24 sec^{-1} or 3.6 sec^{-1} depending upon the molarity of the acetate buffer. Although it might be concluded that acetate ion reacts with Complex II, the data of Fig. 8 show that this is unlikely. In this experiment, the "acceptor" present is nearly exhausted by the addition of only about 3μM peroxide in a total acetate concentration of 10,000 μM. It is more likely that the extent to which "acceptor" becomes available in peroxidase solutions depends upon the type of ions the solution contains.

Table V summarizes the effect of 3 factors upon the transition from the primary to the secondary complex. A 2.6 μM peroxidase was first "stabilized" by about 10 additions of peroxide and then, as the first column of the table shows, the half-time of transition is long (13 sec.) and the velocity constant for the decomposition of the secondary complex is small (0.001 sec^{-1}). But, on diluting a portion of this solution 25 times in 0.01 M phosphate buffer, the transition time and velocity constant correspond approximately to those of an untreated preoxidase as shown in the second column. With 0.001 M phosphate buffer, the velocity constant obtained on dilution of a portion of the "stabilized" solution decreases considerably as shown in Col. 5. Apparently the stronger phosphate buffer increases the amount of "acceptor" available as do both weak or strong acetate buffer.

TABLE V

The Effect of Enzyme Dilution, pH, and Buffer Concentration upon the Stability of the Secondary Peroxidase-H_2O_2 Complex

pH altered by adding H_3PO_4 or NaOH to 0.001 M phosphate buffer pH = 7.0, λ = 427 (cuvettes—Exp. 298)

Enzyme concentration—μM	2.6	0.104	0.104	0.104	0.104	0.104	0.104
H_2O_2 added to test stability—μM	1.4	4	4	4	4	4	4
pH	7.0	7.0	3.65	5.0	7.0	8.8	9.1
Buffer concentration M	0.01	0.01	0.001	0.001	0.001	0.001	0.001
Recorder deflection at 427 mμ	—	—	24	36	49	42	40
Half-time for formation of secondary complex—sec.	13	1.2	0.5	—	1.3	1.7	2
Half-time for decomposition of secondary complex—sec.	560	30	32	70	100	235	>300
$k_3 = \dfrac{x_0}{p_{\max}\, t_{\frac{1}{2}}\ \text{off}}$ sec^{-1}	0.001	0.18	0.24	0.075	0.036	0.016	<0.013

The effect of pH is shown in Cols. 3 to 7, and there is a marked increase of stability of the secondary complex with increasing pH. There is also a decrease of the transition time from the primary to the secondary complex.

From this table, it is seen that optimum conditions for decelerating the transition from the primary to the secondary complex are dilute phosphate buffer at alkaline pH. Under these conditions the minimum amount of "acceptor" is obtained.

In Table VI, a sample of peroxidase has been "stabilized" by 10 additions of H_2O_2 in dilute phosphate buffer, and an extremely slow break-down constant and a long transition are obtained. A similar effect may be obtained with methyl hydrogen peroxide.

The activity of these "stabilized" peroxidase solutions as tested with ascorbic acid does not appear to have been greatly diminished by this treatment.

It has not been possible to obtain such slow transition times using strong peroxidase solutions (~ 100 μM) required for visual spectroscopy, and usually a mixture of Complexes I and II is obtained (7).

TABLE VI

A Comparison of the Effect of Successive Additions of H_2O_2 and Methyl Hydrogen Peroxide upon the Stability of the Enzyme-Substrate Complexes

0.52 μM horseradish peroxidase, pH = 7.0, 0.001 M phosphate buffer (cuvettes—Exp. 294a and 294c)

Substrate	Hydrogen peroxide		Methyl hydrogen peroxide	
Concentration added for each cycle—μM	0.17	0.17	0.34	0.34
Cycle number	1	10	1	6
Recorder deflection at 427 mμ ($e = 32$ divisions)	18	24	23	24
Half-time for formation of complex—sec.	—	10	—	3
Half-time for decomposition of complex—sec.	25	95	11	35
$k_3 = \dfrac{x_e}{p_{max}\, t_{\frac{1}{2}} \text{ off}}$ sec^{-1}	0.022	0.004	0.08	0.025

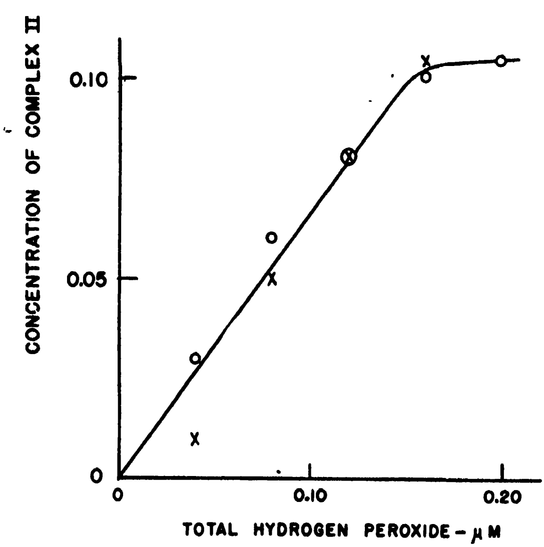

FIG. 9. Illustrating the high affinity of peroxidase for peroxide. A titration of a stabilized peroxidase solution (8 previous additions of 0.4 μM H_2O_2) by rapid addition of increased amounts of H_2O_2 at both 390 mμ (crosses) and 427 mμ (circles). The average value of the dissociation constant is 1.5×10^{-8} M. 0.104 μM horseradish peroxidase, pH = 4.7, 0.01 M acetate buffer (Exp. 167).

These dilute "stabilized" solutions of peroxidase are suitable for a determination of the dissociation constant by direct titration with H_2O_2 as shown in Fig. 9. The titration was carried out at both 390 and 427 mμ. The affinity of peroxidase for H_2O_2 is so high that, even with this very dilute peroxidase solution, the dissociation constant cannot be calculated accurately because the amounts of peroxide complex formed are nearly equal to the amounts of peroxide added. An average value of the dissociation constant (Km_2) is 1.5×10^{-8} M. In this experiment, $k_3 = 0.01$ sec^{-1} and $k_7 = 0.1$ sec^{-1}. From Eq. 12, $Km_2 = \frac{k_3}{k_1}\left(\frac{k_2}{k_7} + 1\right)$ because $k_3/k_7 \ll 1$. Since Km_2 was measured to be 1.5×10^{-8}, k_2 can be calculated to be 1.5 sec^{-1}. This value of k_2 cannot be considered to be accurate because of the inaccuracy of Km_2.

The Effect of pH upon the Dissociation Constant of the Peroxidase-Methyl Hydrogen Peroxide Complex

It is of considerable interest to determine whether there is competition between the peroxide molecule and the hydroxyl group found by Theorell (7), which is believed to occupy the sixth coordination place on the iron atom of peroxidase. Since [Per·OH] of Eq. 1 or e of Eq. 13 would be altered by the dissociation of this hydroxyl group in acid solutions, the value of Km_2 has been calculated according to Eq. 13 on the basis of titrations of peroxidase with peroxide in neutral and acid solutions. Since peroxidase has a lower affinity for methyl hydrogen peroxide than for H_2O_2 or ethyl hydrogen peroxide, methyl hydrogen peroxide has been used to determine the effect of pH upon the dissociation constant of the secondary complex. Fig. 10 clearly shows that there is no effect of the type found by Theorell for fluoride ion, and, to within the accuracy of the data, the dissociation constant does not change from pH = 3.6 to 8.8. Thus, there is no competition between the hydroxyl and peroxide groups for peroxidase hematin; presumably the combination of peroxidase and peroxide follows Eq. 1 in this pH range. At pH 11.3, the optical density change caused by the formation of the red alkaline form of peroxidase was measured, but this form of peroxidase cannot form a peroxide complex.

It is difficult to determine the dissociation constants of these unstable complexes with accuracy, and, therefore, a control experiment on the effect of pH upon the affinity of peroxidase for cyanide was carried out. In a previous paper (10), it was noted that no change occurred between pH = 4.2 and 6.2, but the experiment was repeated with this pure peroxidase preparation as shown in Fig. 11. These data show that peroxidase forms nearly exactly the same amounts of cyanide complex

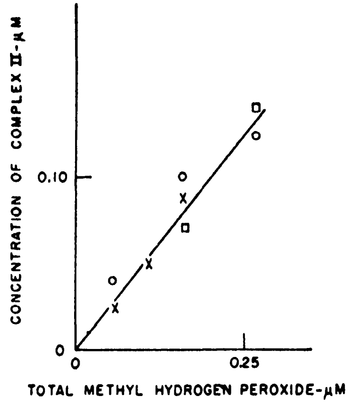

FIG. 10. Illustrating the lack of pH effect upon the affinity of peroxidase for peroxide. A titration of peroxidase with methyl hydrogen peroxide at pH = 3.6 (crosses), pH = 7.0 (circles), and pH = 8.8 (squares). 0.001 *M* phosphate buffer at pH = 7 was acidified with H_3PO_4 to give pH = 3.6. 0.07 *M* borate buffer was used at pH = 8.8. 0.26 μ*M* horseradish peroxidase, 427 mμ (Exp. 294).

at the two pH values and, therefore, the dissociation constant is not altered by this change of pH. The dissociation constant of hydrocyanic acid is 2.06 × 10^{-9}, and therefore a pH change from 4.6 to 7.0 causes a negligible decrease in the concentration of the HCN.

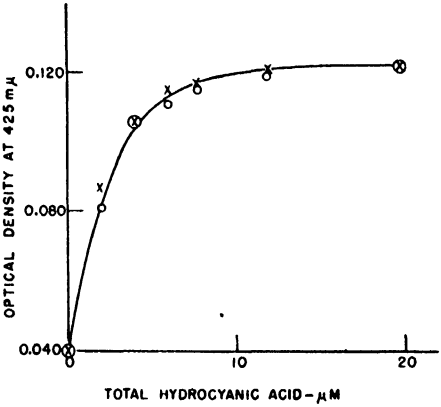

FIG. 11. Illustrating the lack of pH effect upon the affinity of peroxidase for cyanide between pH = 7.0 (circles) and pH = 4.6 (crosses). The latter pH was obtained by acidification with H_3PO_4. 1.67 μ*M* horseradish peroxidase, λ = 425 mμ.

The Acceleration of the Transition from the Primary to the Secondary Complex

Theorell has postulated, on the basis of his visual observations of the slowness of the formation of the red complex, that the green complex is the physiologically important one since the red one would not have time to form during enzyme activity (7,9,11).

In the presence of peroxide alone, there is no indication that the red complex could form rapidly enough to explain peroxidase activity. The values of $k_7 = 4$ sec^{-1} are relatively independent of the peroxide concentration and are quite inadequate to account for the peroxidase turnover of about 2000 sec^{-1} in the P. Z. experiment (12). But the experiments above clearly indicate that the "acceptor" substance increases not only k_3, but also k_7. The effect of known amounts of acceptor upon k_3 and k_7 is demonstrated in Fig. 12.

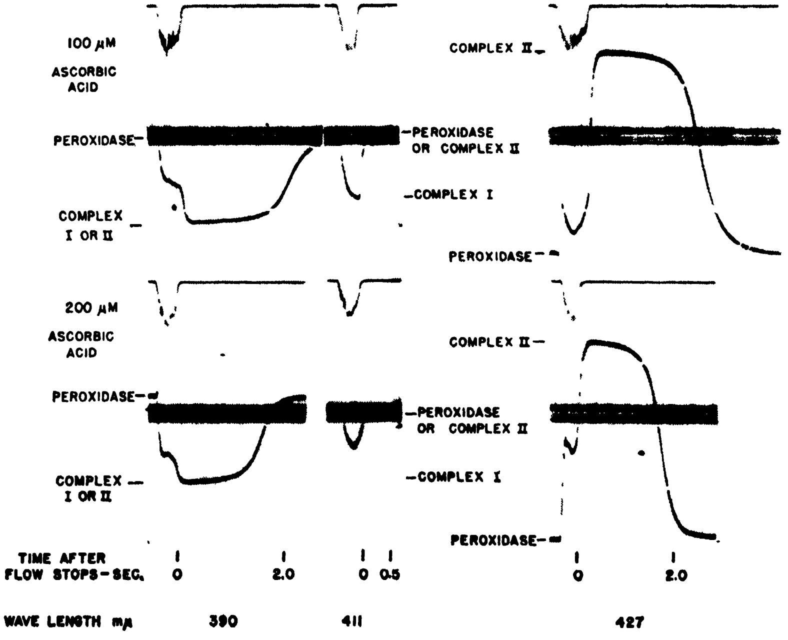

FIG. 12. Illustrating the acceleration of the transition from the primary to the secondary peroxidase-H_2O_2 complex. In the upper set of curves, 100 μ*M* ascorbic acid was present, and in the lower set, 200 μ*M* ascorbic acid was present. Note the more rapid formation of Complex II at 427 mμ and the more rapid disappearance of Complex I at 411 mμ. 2.6 μ*M* horseradish peroxidase, 8.6 μ*M* H_2O_2, pH = 4.6, 0.01 *M* acetate (Exp. 165b).

In the presence of an acceptor such as ascorbic acid,[3] the transition from the primary to the secondary complex is no longer slow; it is a very rapid reaction. In fact, as the ascorbic acid concentration is increased, the primary complex is transformed into the secondary complex almost as rapidly as the primary complex is formed. This effect is clearly shown by the significant changes in the extent of the reaction measured at 411 and 427 mμ during the flow of reactants in the capillary observation tube of the rapid-flow apparatus.

At 390 mμ, it is seen that the increase of ascorbic acid concentration from 100 to 200 μM does not appreciably alter the rate of formation of the primary complex—the extent of reaction at the maximum value of flow velocity is about the same in both cases.

At 427 mμ, the two-fold increase in acceptor concentration causes a very large increase in the velocity with which the secondary complex is formed as shown by the increase of the extent of the reaction at the maximum value of flow velocity. The first-order reaction velocity constant (k_7) for the formation of Complex II calculated from the flow velocity and the extent of reaction during the flow is increased 5-fold—from 26 to 130 sec^{-1}. At 411 mμ, the rate of disappearance of the primary complex is also increased by doubling the ascorbic acid concentration and, at the maximum value of flow velocity, about half of the primary complex has already disappeared to form the secondary complex.

Since the breakdown velocity of the secondary complex increases with ascorbic acid concentration, the duration of the spectroscopic cycle, measured after the flow has stopped, decreases on increasing the ascorbic acid concentration.

Fig. 13 gives the interrelations between the second-order velocity constant for the formation of the primary complex (k_1), the velocity constant for the formation of the secondary complex (k_7) (the values of k_7 are divided by the approximately constant initial H_2O_2 concentration so that they can be compared with the values of k_1), and the breakdown constant for the secondary complex (k_3) for a wide range of ascorbic acid concentrations. As would be expected, the reaction-velocity constant for the formation of the primary complex (k_1) is not much affected by the ascorbic acid concentration. Also, the breakdown constant of the secondary complex (k_3) increases linearly with ascorbic acid concentration as found previously (1). Above 100 μ*M* ascorbic acid, the velocity constant for the formation of the secondary complex also increases linearly with the ascorbic acid concentration and becomes very nearly as rapid as the formation of the primary complex. Thus, the rate of formation of the secondary complex does not become a limiting step in the activity of peroxidase. Below 100 μ*M* ascorbic acid, the "spontaneous" conversion from Complex I to Complex II is sufficiently fast so that the added acceptor causes no measurable acceleration of k_7.

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[3] Ascorbic acid is most convenient because it forms no interfering colored reaction products.

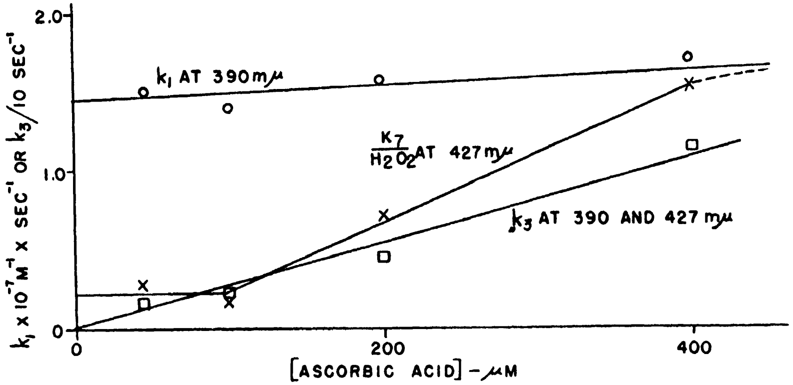

FIG. 13. The effect of acceptor concentration on the three principal velocity constants of peroxidase kinetics (see Eqs. 1, 2, and 3), $k_7/[H_2O_2]$ is plotted on the same scale as k_1. 2.6 μ*M* horseradish peroxidase, 2 6 μ*M* H_2O_2, pH = 4.6, 0.01 *M* acetate buffer (Exp. 165b).

After the initial "flash" of appearance and disappearance of Complex I upon suddenly mixing enzyme and substrate, the concentration of Complex I remains negligible. Except for this initial flash, the kinetics of peroxidase are equivalent to those of a system in which only one intermediate complex exists. And it was on the basis of this assumption that excellent agreement was obtained between the experimentally-determined reaction kinetics of peroxidase and mathematical solutions of the Michaelis theory (1).

The rate-determining step in peroxidase kinetics is usually the reaction of complex II with the acceptor according to Eq. 3. At high acceptor concentrations, the rate of formation of Complex I (approximately equal to the rate of formation of Complex II) or the rate of breakdown of the enzyme-substrate-acceptor complex (12) may become the rate-determining step.

These experiments can be carried out only over a rather narrow range of conditions. First, the peroxide concentrations must be small so that the reaction speeds are within the range of the flow method. Second, the ascorbic acid solutions contain, even at pH = 4.7, an appreciable percentage of H_2O_2 due to autoxidation. This H_2O_2 concentration is determined in a separate experiment where peroxidase is mixed with the ascorbic acid solution alone and the cycle time measured. In these particular experiments, it was found that H_2O_2 equivalent to 5% of the ascorbic acid concentration was present. When using very high ascorbic acid concentrations, this amount of peroxide may be sufficient to make the formation of the complexes too rapid to measure with this apparatus.

This acceleration of the transition from Complex I to II has been found to occur with all acceptors with which peroxidase reacts. Ascorbic acid is used to obtain quantitative data over the widest range of experimental conditions. The exact relation between k_7 and the concentration of acceptors other than ascorbic acid is often difficult to investigate because of the formation of colored oxidation products.

DISCUSSION

The velocity constant for the formation of peroxidase-H_2O_2-I has been remeasured by these improved techniques and is found to be $0.90 \pm 0.2 \times 10^7\ M^{-1} \times \text{sec}^{-1}$ compared with $1.2 \pm 0.4 \times 10^7\ M^{-1} \times \text{sec}^{-1}$ (1) found earlier. The velocity constant for the formation of the primary compounds of peroxidase with methyl or ethyl hydrogen peroxide are found to be 1.5 and $3.6 \times 10^6\ M^{-1} \times \text{sec}^{-1}$, respectively. As contrasted to catalase (6), there is no systematic decrease of the reaction velocity constant with the size of the substrate molecule. The reactivity of peroxidase with large acceptor molecules, and the ease with which peroxidase is split into hemin and protein components suggest that the hematin group of peroxidase is more accessible than that of catalase (6, 13).

All three of these primary complexes rapidly shift to the red secondary form at a speed which appears to be directly proportional to the concentration of the primary complex. The formation of the secondary compound is, therefore, caused by a first-order decomposition of the primary compound. The primary complex is apparently quantitatively converted into the secondary complex; the velocity of the reverse reaction is negligible under these experimental conditions.

There appears to be proportionality between the rate of decomposition of the primary compound and the rate of formation and the rate of decomposition of the secondary compound. In other words, if the breakdown rate of the secondary compound is decreased by some of the methods discussed in the paragraph below, then both the rate of disappearance of the primary complex and the rate of appearance of the secondary complex are slowed.

These 3 reactions may be slowed by adding several equivalents of peroxide to the peroxidase solution. The slowing of these reactions does not, however, cause any loss of activity when tested in the usual manner. It is postulated that peroxidase contains an acceptor which is released to varying degrees, depending upon the pH, type of buffer solution, dilution of the enzyme, and probably many other factors. Peroxidase in strong acetate buffer contains much more acceptor than in dilute alkaline phosphate buffer. The small amount of peroxide required to remove this acceptor shows that the acceptor is present in a concentration of the same order of magnitude as the enzyme. The effect of such a small amount of acceptor upon the value of k_3 suggests that this substance reacts as rapidly with Complex II as would pyrogallol. Whether this acceptor is a foreign substance adsorbed to peroxidase, or is actually a dissociable component of peroxidase, is a question which these experiments do not answer.

The dissociation constant of the secondary peroxidase-H_2O_2 complex is so small that it is difficult to measure accurately as has been found before (1). The ratio k_3/k_1 is between 1 and 3×10^{-9} M for untreated peroxidase solutions. Titration of "stabilized" peroxidase with peroxide gives dissociation constants which may be as large as 1.5×10^{-8} M using an enzyme solution which is as dilute as possible (1×10^{-7} M). Thus, it is possible that the reversible dissociation velocity (k_2 of Eq. 1) may be as large as 1.4 sec^{-1}. With methyl hydrogen peroxide, a value of $k_2 = 2.2$–3.4 sec^{-1} is also possible. One objection to the existence of values of k_2 as large as 3 sec^{-1} is the fact that the values of k_1 calculated as if $k_2 = 0$ are unchanged as the initial peroxide concentration is decreased (see Tables I, II, III). In fact, this larger value of k_2 is the only velocity constant for peroxidase action which differs significantly from those values found previously in studies based on Complex II (1). However, the experimental error of the titration data is large and, thus, the existence of k_2 is not yet fully established.

Theoretically, k_2 could represent the velocity constant for the dissociation of either Complex I or Complex II. But kinetic data show that Complex I is quantitatively converted into Complex II. Therefore, k_2 is taken to represent the velocity constant for the dissociation of the primary complex.

On addition of acceptor to Complex I, its transition to Complex II is speeded, as is the decomposition of the latter. Thus, the action of the acceptor is not upon Complex II alone. But the strict proportionality (over a reasonable range) between acceptor concentration and the rate of decomposition of Complex II supports the simple second-order reaction of acceptor and Complex II shown in Eq. 3. If the reaction of acceptor were with Complex I only, it would be difficult to see how the rate of breakdown of Complex II could vary in a second-order fashion with acceptor concentration. This most important portion of the enzyme mechanism requires more penetrating experimentation.

However, it is now possible to explain two previous and apparently contradictory results: (1) Theorell's postulate that his green Complex I was the only one of physiological importance (7,9,11) and (2) Chance's studies of the kinetics of peroxidase with an apparatus sensitive to Complex I (1).[4]

Theorell's conclusion, based upon the apparently sluggish conversion of Complex I to Complex II observed in strong peroxidase solutions, must be revised in view of these findings that the speed of transition may greatly increase in the presence of acceptors and the transition may be complete in a few milliseconds. Thus, both the green primary complex and the red secondary complex are involved in the activity of peroxidase.

In the presence of acceptor, the transition from Complex I to Complex II is much faster than the reaction of the latter with the acceptor; Complex II is the rate-determining intermediate in peroxidase activity. Thus, the previous studies (1) of the kinetics of Complex II as the Michaelis complex in peroxidase activity are validated by these data.

These same findings validate the earlier studies on the reaction kinetics (1). The half-time for the formation of Complex II from Complex I with that peroxidase preparation is estimated to be on the order of 0.02 sec. in the absence of added acceptor. Thus, the kinetics of formation of Complex II accurately represent those of Complex I in dilute H_2O_2 solutions (when $k_7 > k_1\,[H_2O_2]$) and the values of k_1 are in agreement with those obtained in this paper. As mentioned in that paper, deviations from the second-order reaction were obtained, as would be expected, when k_7 is about equal to $k_1[H_2O_2]$.

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[4] The apparatus was a differential colorimeter measuring the difference of optical density at 390 and 420 mμ and was, therefore, much more sensitive to the spectral shift from peroxidase to Complex II than to Complex I.

The dissociation constant of peroxidase-methyl hydrogen peroxide is found to vary but little from pH = 3.6 to 8.8. Similar constancy of the dissociation constant of peroxidase cyanide is observed from pH = 4.6 to 7.0. Later data show that the activity of peroxidase with some acceptors varies but little between pH 6.5 and 3.8 (12).

Theorell and Paul (4) have found two heme-linked groups in peroxidase having $pK = 4.0$ and 5.0. They concluded that the former is caused by the dissociation of the iron-protein linkage and the latter is caused by the dissociation of a hydroxyl group bound to the iron atom of peroxidase at the sixth coordination position.

In the pH range from 8.8 to 5, peroxidase is mainly in the form of the hydroxide compound (4) and, as suggested by studies of catalase (6,14), the HCN or peroxide molecules combine with peroxidase hydroxide as follows:

$$\text{Per}\cdot\text{OH} + \text{HOOR} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{Per}\cdot\text{OOR} + H_2O. \qquad (15)$$

This is an exchange reaction between the hydroxyl and peroxide ions and no effect of pH would be expected, in accordance with the experimental data.

Below pH 5, one would expect large changes in the dissociation constant of the peroxidase-peroxide complexes caused by the dissociation of the two heme-linked groups of $pK = 4$ and 5 (4). But no change in the dissociation constant of either peroxidase-peroxides or peroxidase cyanide has been found at these values of pH. According to these data, the reaction represented by Eq. 15 applies to the reactions of peroxidase with both cyanide and peroxide up to about pH 3.6. It is possible that the hydroxyl group does not dissociate from peroxidase hematin at the value $pK = 5.0$ under these experimental conditions. A further discussion of this effect is to be given in another paper.

Above pH = 9, peroxidase changes to the red alkaline form where a hydroxyl group is bound to the iron atom by covalent bonds (7). Peroxides are no longer able to replace the hydroxyl group and do not combine with this molecule. Peroxidase is, therefore, inactive in its alkaline form.

Since Complex II has covalent bonds (7,5) and is directly involved in the enzymatic activity, there is no support for the generalization that only compounds of enzyme and substrate with ionic bonds can react rapidly enough to be intermediates in enzymatic activity (7,9). Nevertheless, the similarity of the spectra of the *primary* complexes of catalase and peroxidase (3,5) is so great that they surely have the same bond type, which is probably ionic. Thus, there is considerable support for the generalization that these primary, and probably ionic, compounds of enzyme and substrate form first and more rapidly than covalent compounds.

In peroxidase, a covalent complex is formed before the oxidation of the acceptor occurs. Thus far, experiments with catalase indicate that the primary complex reacts directly with acceptors; the shift to a covalent complex is not required (6). However, it is possible that a catalase-peroxide complex (in addition to the known inactive secondary complex (6)) analogous to the catalytically active secondary peroxidase complex exists, but its reaction with acceptors is more rapid than the primary to secondary transition—*i.e.*, $k_7 \ll k_3$ in Eqs. 2 and 3, and k_7 varies with acceptor concentration as found with peroxidase. It is not yet possible to carry out an experiment which would decide this question with certainty.

SUMMARY AND CONCLUSIONS[5]

1. The primary complexes of peroxidase and peroxides are formed in a rapid second-order reaction. The combination with H_2O_2 is the most rapid, $k_1 = 0.90 \pm 0.2 \times 10^7 M^{-1} \times \text{sec}^{-1}$ (compared with $1.2 \pm 0.4 \times 10^7$ M^{-1} sec^{-1} obtained previously (1)). The combination with the alkyl hydrogen peroxides is slower, 1.5×10^6 $M^{-1} \times \text{sec}^{-1}$ for methyl hydrogen peroxide, and $3.6 \times 10^6 M^{-1} \times \text{sec}^{-1}$ for ethyl hydrogen peroxide.

2. The secondary peroxidase-peroxide complexes are formed from the primary complexes in a first-order reaction which, under usual conditions, has a velocity constant of about 4 sec^{-1}. Under these conditions, the primary complex is quantitatively converted into the secondary complex.

3. The secondary complexes decompose spontaneously at rates which depend principally upon whether peroxide has been previously

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[5] These velocity constants were measured between 25° and 30°C.

added to the solution. Untreated peroxidase solutions give velocity constants of about 0.02 sec^{-1}, but several additions of peroxide to peroxidase solution can reduce this velocity constant by a factor of ten. To explain this effect, it is postulated that the enzyme is accompanied by an acceptor substance wihch accelerates the decomposition of the secondary complexes. This substance is present in the smallest concentrations in phosphate buffer at alkaline pH and can be removed by peroxidatic oxidation upon the addition of peroxide. This substance may be present in a concentration somewhat greater than that of the enzyme.

4. The rate of conversion of the primary to the secondary complexes is related to the rate of decomposition of the secondary complex and, therefore, the primary complexes have the longest lives when the substance above has a minimum concentration.

5. The dissociation constant of the secondary peroxidase-H_2O_2 complex is about 1.5×10^{-8} M and is somewhat greater than the ratio of the rate of decomposition of the secondary complex (k_3) to the rate of formation of the primary complex (k_1) ($k_3/k_1 \approx 2 \times 10^{-9}$ M). It is possible that a reversible decomposition of the primary or secondary complexes at the rate of 1.4 sec^{-1}, or less, occurs.

6. The dissociation constant of the primary peroxidase-methyl hydrogen peroxide complex has been measured and, for the particular experimental conditions, has been found to be 3.2×10^{-6} M. This is in agreement with the value obtained from kinetic data provided k_2 is assumed to be 2.2 sec^{-1}. The dissociation constant of the secondary complex is about 3×10^{-7} M and is in agreement with kinetic data provided k_2 is assumed to be 3.4 sec^{-1}. The evidence for the existence of a value of k_2 of this magnitude is regarded as preliminary.

7. The dissociation constant of peroxidase-methyl hydrogen peroxide has been found to be little affected by pH in the range 3.6 to 8.8. Neither of the two heme-linked groups found by Theorell and Paul (4) appears to affect the ability of peroxidase to form its enzyme-substrate complexes.

8. The lack of an effect of pH upon the dissociation constant of the peroxidase-methyl hydrogen preoxide complex suggests in the region between 8.8 and 3.6 that peroxidase and peroxide combine as follows:

$$\text{Per}\cdot\text{OH} + \text{HOOR} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Per}\cdot\text{OOR} + H_2O.$$

9. The formation of the alkaline form of peroxidase prevents the formation of the peroxide complexes. This covalent compound does not react with peroxides.

10. Contrary to Theorell's postulate (7,9,11), the enzymatic activity of peroxidase involves both the green primary and the red secondary complexes. The transition from the primary and the red secondary complex is accelerated by the presence of an acceptor and the velocity of formation of the secondary complex from the primary complex can be nearly as rapid as the formation of the primary complex.

11. Since the red secondary complex probably has covalent bonds (7), covalent compounds of enzyme and substrate are here shown to be enzymatically active.

12. The enzyme-substrate complex studied previously (1) is the red secondary complex, and these experiments support the conclusion that this complex is the rate-determining, or Michaelis intermediate, compound in peroxidase action; the steady-state concentration of the primary complex in peroxidatic reactions is very small.

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A Polysaccharide Related to the Blood Group Substances and its Reaction with Borate. I. A Study by Electrophoresis

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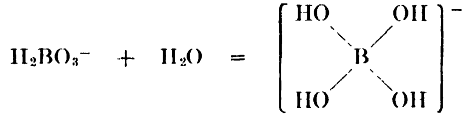
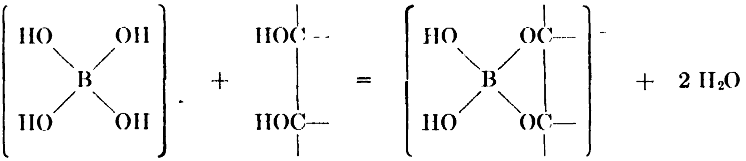
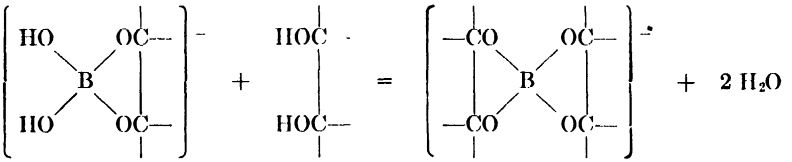
INTRODUCTION

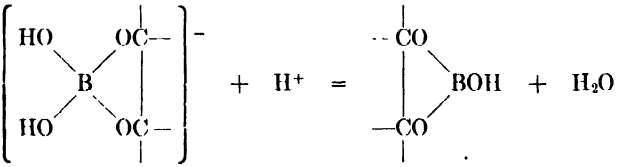
Sugars and other polyhydroxy compounds with adjacent hydroxyl groups in the *cis* position react with borate solutions to form coordination compounds of increased acidity and optical activity (1,2,3):


(a)


(b)


(c)

The mono-diol structure (b) is favored by low concentrations of diol and low ratios of diol to borate, the more strongly acidic (4) di-diol configuration (c) by high concentrations of diol and high ratios of diol to borate. On addition of acid the compounds are either decomposed, with regeneration of the sugar and formation of a salt and boric acid (5), or converted to a cyclic boron compound of weak acidity (1):



(d)

The reaction of borates with polysaccharides containing the required configuration (6) is accompanied by further physical changes because of the formation of multiple di-diol borate linkages between adjacent polysaccharide chains. These linkages, which are continually being severed and restored (6), produce a labile network characterized by gel formation (6), increased viscosity (7,8), and altered precipitability by neutral salts (8). The augmented ionization increases the electrophoretic mobility (8,9,10).

A polysaccharide isolated from calf intestinal mucosa (8,9,11) exhibits all the above porperties in the presence of borate. It is serologically related to the blood group A substance, which also reacts with borate (9,11) and which is known to contain two sugars, L-fucose and D-galactose, with the required configuration (12). The electrophoretic mobility of the polysaccharide was determined in borate-free solutions and in solutions in which the buffer salts were replaced, either wholly or partially, by borate-boric acid mixtures. Increase in mobility was dependent upon three factors, pH, concentration of borate ion, and concentration of polysaccharide, and was paralleled by a sharpening of the electrophoresis boundaries.

MATERIALS AND METHODS

The two preparations of polysaccharide investigated have already been described as to method of preparation and chemical and serological properties (9). One (I) gave a single boundary in borate-free solution [Fig. 1 of (9)], the other (II) contained 10–15% of slowly migrating constituents (Fig. 2).

Hexuronic acid determinations by the method of Dische (13) were negative with even 100 γ of preparation II. A sample of hyaluronic acid gave an average reading, close to that reported by Dische, of 200/100 γ of sample for the range 10–100 γ in the Klett-Summerson photoelectric colorimeter.

The HCl hydrolyzates prepared for determination of the reducing sugars (9) were analyzed gravimetrically for sulfate content by microprecipitation with $BaCl_2$; an average value of 1.5% was obtained. The phosphorus content, determined by a slight modification of the King colorimetric method, was 0.26%.

Electrophoresis was carried out at an ionic strength of 0.20. Buffering action was provided by mixtures of mono- and disodium phosphates and mixtures of H_3BO_3 and $Na_2B_4O_7$ (one mol of which in dilute solution (14) dissociates into two mols of sodium borate and two mols of boric acid), each supplemented when necessary by citric or hydrochloric acids or NaOH. In the phosphate buffers, 0.15 of the ionic strength was contributed by NaCl and 0.05 by buffer salts. This would have necessitated 0.05 *M* sodium borate in all borate buffers, and led to excessive concentrations of boric acid below pH 8 (15). Instead, the combined concentrations of sodium borate and boric acid were made equal to 0.20 *M* below pH 8.6, and the concentration of NaCl adjusted to maintain an ionic strength of 0.20.

The polysaccharide solutions, ordinarily containing 10 mg./ml., were made up from weighed quantities of the dry substance and dialyzed 16 hr. Electrophoresis was carried out at a potential gradient of about 5 V/cm. in the Tiselius apparatus (16). The specific refractive increment computed from the curve areas was 0.0014. The descending and ascending boundaries gave concordant values for the mobilities.

PRESENTATION AND DISCUSSION OF RESULTS

Mobility in Borate-Free Solution

The mobility of the polysaccharide as a function of pH is shown by the solid lines of Fig. 1. Preparation I (lower curve) had a constant mobility of -3.3×10^{-5} cm./sec. volt/cm. from pH 8.6 to about 4.5; below pH 4.5 the mobility diminished, but migration was still anodic at pH 1.5. The mobility of preparation II varied with pH, and was less

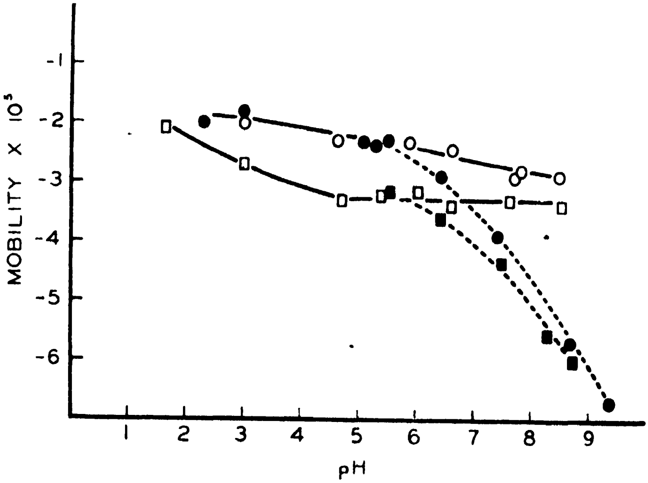


FIG. 1. Mobility of the polysaccharide as a function of pH. □ Preparation I in borate-free solution; ○ Preparation II in borate-free solution; ■ Preparation I in borate solution; ● preparation II in borate solution.

than that of preparation I; the slowly migrating constituents contaminating preparation II may have been responsible for this difference through reversibly dissociable combination (17). The negative mobility at low pH values is probably, as in heparin (18), caused by ionization of sulfate groups. Sulfate groups have been found in other perparations of blood group substance (19).

Mobility in Borate Solutions

The black symbols and dotted lines in Fig. 1 refer to experiments in borate-containing solutions. The mobility of the polysaccharide was unchanged in the pH range 5.5 and below, where ionization of boric acid is negligible (15), but increased rapidly above pH 5.5. The rise in mobility was paralleled by a sharpening of the boundaries (Fig. 2a), so that, at high pH values, each preparation gave a single sharp peak.

In the presence of borate ions some of the diol groups in each polysaccharide molecule are combined as the mono-diol complex, some as the di-diol complex, and the rest free. Of the complexes, some are ionized, and the remainder neutralized by hydrogen ions; the former determine the mobility increment. The configuration of each molecule must shift continually (6), but, because of the swift establishment of equilibrium, any configuration differing radically from the average in its proportions of bound and charged diol groups will have only a transient existence (20).

The diol-borate complexes are stronger acids than boric acid, with lower *pK* values (15). They might be expected to show appreciable ionic dissociation, and cause a slight rise in mobility, in a short pH range below the lower limit [pH 5.2 (15)] of the boric acid titration curve. In solutions containing no sodium borate, however, the concentration of the complex borate ion (a) formed through dissociation of boric acid alone is small, and the tendency for combination with diols is slight. Sufficient combination with diols does occur to cause measurable changes in acidity (1,3,14,15), but the concentrations of the complexes, both ionized and non-ionized, are too low to detect by optical rotation methods (3). The failure of borate to change the mobility of the polysaccharide below pH 5.5 may thus be ascribed to inadequate concentrations of the ionized form of the complexes, a result of the combined effects of limited combination and low degree of ionization. Above pH 5.5 the sodium borate concentration increased with pH in

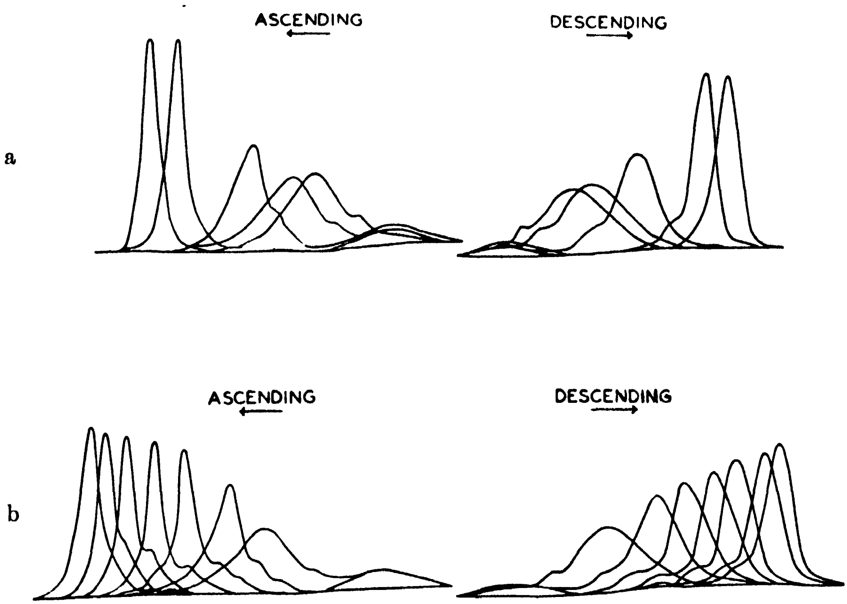


FIG. 2. Tracings of Svensson diagrams for preparation II. a. Electrophoresis for 3 hr. in borate solution, showing the effect of pH on the shape and movement of the boundaries. From left to right, descending patterns, the pH values are 5.5, 6.4, 7.4, 8.6, and 9.4, respectively. b. Electrophoresis for 4 hr. at pH 8.6, showing the effect of sodium borate concentration on the shape and movement of the boundaries. From left to right, descending patterns, the sodium borate concentrations are 0, $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, and 1 × 0.05 *M*, respectively.

the manner indicated by the boric acid titration curve (15) to a value of 0.05 *M* at pH 8.6. The increased combination of borate with diol induced by the mounting concentration of borate ion, and the greater ionic dissociation of the complexes resulting from rise in pH, were together responsible for the rapid rise in mobility above pH 5.5.

Variation of Borate Concentration at Constant pH and Polysaccharide Concentration

To separate the effects of borate ion concentration (which determined the nature and extent of diol-borate combination) from that of pH (which determined the degree of ionic dissociation of the diol-borate complexes), preparation II of the polysaccharide was studied in solutions of varying borate concentration and constant pH. The solvents were prepared by combining the borate-containing buffer of pH 8.6 with the borate-free buffer of the same pH to give the dilutions 1/16, 1/8, 1/4, 1/2, and 3/4. The polysaccharide boundaries were accelerated and sharpened as before (Fig. 2b), but the greatest changes were produced by the initial, rather than the final, increments of sodium borate. The difference is shown in Fig. 3, in which the mobility increments (●) at pH 8.6 and pH 7.4 are plotted as a function of sodium borate concentration. The increment at pH 7.4 was less, because of lower ionization of the complexes, than that produced by the same concentration of sodium borate (*ca.* 0.0125 *M*) at pH 8.6. The further change in mobility on increasing the borate concentration to 0.05 *M* was thus greater for the former, the pH of which was simultaneously increased from 7.4 to 8.6, than for the latter, the pH of which remained the same.

The striking ability of low concentrations of borate to augment the mobility of the polysaccharide, and the decline in the effectiveness of successive increments of borate, may be explained by the predominance of the di-diol over the mono-diol structure at low borate concentrations, and the gradual increase in the proportion of the mono-diol structure with rising borate concentration and falling diol-borate ratio. Borate ions which are combined in the di-diol configuration convert two diol groups to ionizable form and augment the mobility of two polysaccharide molecules, while those combined in the mono-diol configuration affect only one diol group. The predominance of the di-diol configuration at low borate concentrations should thus cause the concentration of ionizable diol groups to mount most readily in this range. The strongly acidic character of the di-diol structure as compared with the mono-diol, and the lower equilibrium concentration of free borate ion when diols are in excess, probably contribute also to the observed changes. The flatness of the curve from 0.0375 to 0.05 *M* sodium borate (Fig. 3) suggests relatively complete conversion of the di-diol to the mono-diol borate configuration at 0.05 *M*.

Variation of Polysaccharide Concentration at Constant pH and Borate Concentration

The nature and extent of diol-borate combination are influenced by the absolute concentration of diol as well as by the ratio of this concentration to that of borate ion. By mass action principles, low concentra-

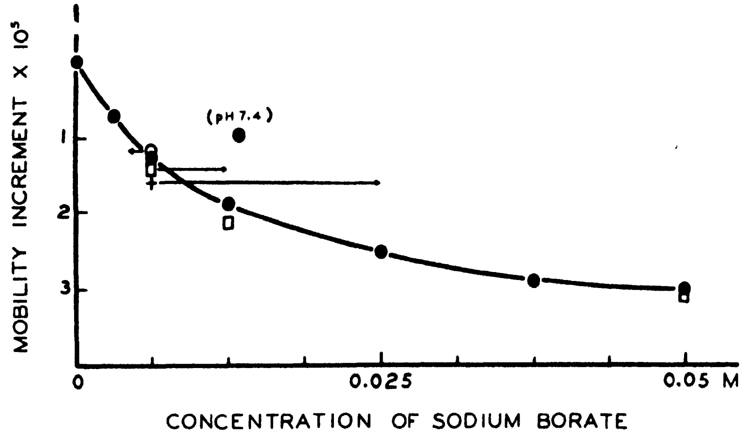


FIG. 3. Mobility increments of Preparation II at pH 8.6 as a function of sodium borate concentration. ○ 15.0 mg. polysaccharide/ml.; ● 10.0 mg. polysaccharide/ml.; □ 5.0 mg. polysaccharide/ml.; + 2.5 mg. polysaccharide/ml. Horizontal lines: see text.

tions of diol should favor the mono-diol structure, and high concentrations the di-diol except at very high borate concentrations.

The mobility increments for solutions containing 2.5 (+), 5.0 (□), and 15.0 (○) mg./ml. are plotted as a function of sodium borate concentration in Fig. 3. At 0.00625 *M* the increment appeared to be greater the lower the polysaccharide concentration. Qualitatively, this finding is reasonable in view of the decreased ratio of diol to borate resulting from the reduction of polysaccharide concentration. Quantitatively, however, as shown below, the increments differed less than would be expected from the changes in this ratio, and indicated the influence of absolute concentration.

From each of the points corresponding to these increments (Fig. 3) a horizontal line has been drawn to the concentration of sodium borate required to produce the same diol-borate ratio in a polysaccharide solution containing 10 mg./ml. If this ratio were the only factor influencing diol-borate combination, the ends of these lines should fall on the curve. Those for the more dilute solutions, however, fall above, and that for the more concentrated below. It thus appears that, for solutions of the same diol-borate ratio, the mobility increment is diminished by dilution. The diminution may be explained by the lower incidence of diol-borate combination, particularly of the strongly acidic di-diol structure, which involves three entities, as the result of lower collision frequency.

Boundary Sharpness

The boundary sharpness was greatest at the high concentrations of sodium borate (Fig. 2) which favor the mono-diol structure (21); it cannot, therefore, be attributed to the formation, through di-diol borate linkage, of large aggregates of increased viscosity (8), reduced diffusion, and greater uniformity of charge. In solutions of approximately equal sodium borate concentrations (see Fig. 3) the boundaries were sharper at pH 8.6 (Fig. 2b, middle diagram) than at pH 7.4 (Fig. 2a, middle diagram). The influence of pH points to an electrical, rather than a molecular kinetic, basis for the sharpening, and suggests the following hypothesis.

The broadness of the polysaccharide boundary in borate-free solution may indicate electrical heterogeneity caused by variation in the content of sulfate or other charged groups. Combination with the quadricovalent borate ion (a) may be impeded by these groups through electrostatic repulsion or steric hindrance. If so, the charge increment resulting from combination with borate ion would be an inverse function of the initial charge on the molecule. The charge heterogeneity of the polysaccharide would then diminish with rising borate ion concentration, for the effect of obstruction by the sulfate groups would increase with competition by the borate ions for the remaining diol groups. It would also diminish with pH, for the variation in the individual charge increments, like the mean charge increment, would depend upon the degree of ionic dissociation of the complexes.

SUMMARY

A polysaccharide isolated from calf intestinal mucosa, and related serologically to the blood group A and B substances, exhibits increased mobility in the presence of borate ions through formation of diol-borate compounds of increased acidity. The mobility increments were found to vary with (1) pH, (2) borate ion concentration, and (3) polysaccharide concentration. Together, (2) and (3) determine the concentrations of the diol-borate compounds; and (1) determines the degree of ionization of those compounds. A sharpening of the electrophoresis boundaries paralleled the rise in mobility.

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A Polysaccharide Related to the Blood Group Substances and its Reaction with Borate. II. A Study by Sedimentation and Viscosity

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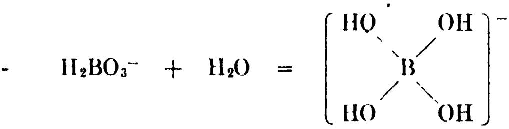
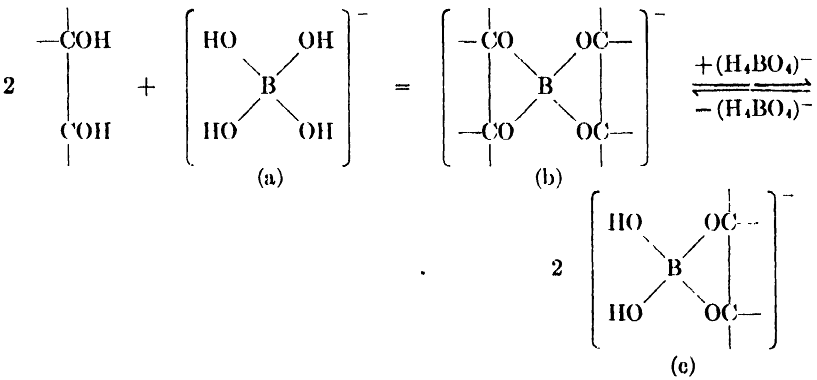
INTRODUCTION

A polysaccharide isolated from calf intestinal mucosa, and closely related to the specific blood group A and B substances, displays increased mobility in the presence of borate ion (1,2,3,4). The rise in mobility depends upon the presence in the molecules of sugar residues containing adjacent hydroxyl groups in the *cis* position, and the reaction of those diol groups with borate ion to form coordination complexes of increased acidity (5):



(1)



(2)

The di-diol borate structure (b), which is the more strongly acidic, is favored by high ratios of diol to borate and high concentrations of diol, and the mono-diol borate structure (c) by low ratios of diol to borate and low concentrations of diol.

The di-diol borate configuration leads to the formation of multiple borate cross-linkages between adjacent polysaccharide chains. The large structures made possible by di-diol borate combination, and the increase in weight resulting from mono-diol borate combination, might be expected to change the sedimentation behavior of the polysaccharide. In view of the transience of the di-diol borate linkages and the rapid establishment of equilibrium between the various types of structure (6,4), however, no sorting of different sizes should occur (7). Instead, the polysaccharide should sediment with a single boundary at a rate determined by the relative concentrations of the units of various magnitudes (7).

Sedimentation studies, paralleled by viscosity measurements, were made on solutions of constant pH in which polysaccharide content and sodium borate concentration were systematically varied. The changes in sedimentation behavior and viscosity produced by addition of borate were smaller and less striking than the changes in electrophoretic behavior, but led to the same interpretation in terms of the proportions of the two types of diol-borate structure.

EXPERIMENTAL

The polysaccharide used for these experiments [preparation II of (3) and (4)] has already been described with respect to chemical, serological, and electrophoretic properties. Borate-free and borate-containing buffer solutions of pH 8.6 were prepared containing 0.15 *M* NaCl and sufficient buffer salts (phosphates and borates, respectively) to raise the ionic strength to 0.20. Portions of the borate buffer, which contained 0.05 *M* sodium borate, were combined with portions of the phosphate buffer to give sodium borate concentrations of 0.025 *M*, 0.0125 *M*, 0.00625 *M*, and 0.0031 *M*. The polysaccharide solutions, containing 2.5, 5.0, 10.0, and 15.0 mg./ml., were either made up directly from weighed quantities of the dried substance, or prepared by dilution or mixing of such solutions. A few measurements were made on solutions which had been prepared for electrophoresis; these had been dialyzed.

Sedimentation measurements were made at 24–30°C. at 42–43000 r.p.m. in a Beams type analytical ultracentrifuge (8,9,10) equipped with the Philpot-Svensson optical system. Viscosity measurements in the Ostwald viscometer and density measurements by the falling drop method of Barbour and Hamilton (11) were made at 30°C. A partial specific volume of 0.7 for the polysaccharide was computed from the specific volumes of the solution and solvent (12).

Viscosity

Sodium borate (Table I) reduced the relative viscosity of solutions containing 2.5 or 5.0 mg. of polysaccharide/ml. At a concentration of 0.05 *M* it reduced the viscosity of polysaccharide solutions containing 10 or 15 mg./ml. as well, but at lower concentrations caused an increase of viscosity, greater at 15 mg./ml. than at 10 mg./ml. The high diol concentrations and high diol-borate ratios of these latter solutions should favor the di-diol borate structure (b); the viscosity increase, therefore, may probably be attributed to structural anomalous viscosity (13), a result of transient di-diol borate linkages between adjacent polysaccharide chains. The reduced viscosity in solutions of lower diol concentration, or of high diol concentration but low diol-borate ratio, may possibly be explained by a diminution in axial ratio caused by increase in effective diameter through conversion of the diol groups to the mono-diol borate complex (c). The viscosity changes in the more concentrated solutions were consistent with Zittle's observation that low concentrations of borate cause gelation of gala gum while high concentrations do not (14).

TABLE I

Variation of Relative Viscosity with Sodium Borate Concentration and Polysaccharide Concentration

Polysaccharide conc., mg./ml.		2.5	5.0	10.0	15.0
Sodium borate in solvent:	0.0000 *M*	1.17	1.38	1.75	2.26
	0.0031 *M*	1.15	1.34	—	—
	0.0062 *M*	1.15	1.33	1.77	2.57
	0.0125 *M*	1.14	1.33	1.73	2.56
	0.0250 *M*	1.14	1.32	1.79	2.68
	0.0500 *M*	1.13	1.27	1.66	2.20

The volume intrinsic viscosity of the polysaccharide in borate-free solution was 78.5; in 0.05 *M* sodium borate (computed on the basis of the weight and partial specific volume of the borate-free polysaccharide) it was 73.5.

Sedimentation

Regardless of the borate content of the solvent, the sedimentation diagram of the polysaccharide consisted of a single boundary, a manifestation of the rapid establishment of equilibrium between the sedimenting units with respect to mono-diol borate, di-diol borate, and uncombined diol structure. The changes in the proportions of each caused by variation in borate content produced small changes in the sedimentation rate, but did not, as in electrophoresis (4), noticeably alter the shape or sharpness of the boundary. This is futher evidence that the sharpening of the electrophoresis boundaries in the presence of borate resulted from changes in the electrical, rather than the molecular kinetic, properties of the molecule (4).

The sedimentation constants were corrected to a basis of sedimentation in water at 20°C. by the conventional method, employing the viscosity and density of the solvent (s_{20}) (15), and also by use of the viscosity and density of the solution (s'_{20}) (16). The latter correction, based on the concept that, for ideal solutions, the sedimentation constant varies as the reciprocal of the solution viscosity (17), was the more useful in interpreting the data. The values obtained by both types of correction are listed in Table II together with the sedimentation constants at infinite dilution determined from the s_{20} values by the extrapolation method of Gralén (18).

In each solvent the sedimentation constants obtained by the conventional correction (s_{20}) showed the strong dependence on polysaccharide concentration which is characteristic of thread-like molecules. In borate-free solution and in 0.05 *M* sodium borate, correction of the sedimentation constant for the solution viscosity (s'_{20}) removed this dependence at polysaccharide concentrations of 10 mg./ml. or less, but at 15 mg./ml. overcompensated it. At this concentration, interference of the polysaccharide molecules may be postulated.

TABLE II

Variation of Sedimentation Constant with Sodium Borate Concentration and Polysaccharide Concentration

	s_{20} (corrected for density and viscosity of solvent)				s_{20} extrapolated (18)	s'_{20} (corrected for density and viscosity of solution)			
Polysaccharide conc., mg./ml.. ...	15.0	10.0	5.0	2.5	0	2.5	5.0	10.0	15.0
Na borate concn.: 0.0000 *M*	3.52	4.03	5.28 5.41	6.09 6.12	7.05	7.16 7.17	7.17 7.23	7.00	7.94
0.0031 *M*	—	—	5.70	6.32	7.20	7.30	7.65	—	—
0.0062 *M*	3.98 4.05 4.18	4.70 4.76	6.08 5.77 5.93	6.55 6.68 6.75	7.62	7.56 7.65 7.80	8.18 7.69 7.89	8.32 8.44	10.26 10.41 10.76
0.0125 *M*	4.03	4.82	6.16 5.92	6.53 6.64	7.70	7.43 7.56	8.22 8.03	8.47	10.47
0.0250 *M*	4.25	5.01	6.16	6.86	7.80	7.83	8.15	8.93	11.37
0.0500 *M*	4.27	4.87	6.80 5.67 6.24	7.00 7.00 6.91	8.00	8.03 7.89 8.18	8.05 7.86 7.97	8.12	9.48

At intermediate concentrations of sodium borate the concentration dependence of the sedimentation constant was overcompensated by the solution viscosity, even at concentrations of 5 mg./ml. It appears that in these solutions transient di-diol borate linkages exerted an effect similar to molecular interference. Since the viscosities at

5 mg./ml. were lower than in borate-free solution, however, the apparent overcorrection must be ascribed to an actual slight increase in sedimentation rate through formation of di-diol borate linkages. If the increase of s'_{20} above the extrapolated value of s_{20} (corrected for the interference of the polysaccharide molecules themselves at 15 mg./ml.) be considered an index of di-diol borate combination, then it appears that little occurred when the polysaccharide concentration was 2.5 mg./ml., regardless of the borate concentration. In 0.0031–0.025 *M* sodium borate di-diol borate combination increased rapidly with polysaccharide concentration. At polysaccharide concentrations of 5 mg./ml. or greater, it rose steeply with sodium borate concentration up to 0.0062 *M*, but with further increase to 0.025 *M* it leveled off, and at 0.05 *M* was insufficient for detection, except, perhaps at 15 mg./ml.

These conclusions are consistent with those drawn from the viscosity data alone, except that the viscosity gave no indication of di-diol borate combination below 10 mg./ml. Sedimentation, however, should be a more sensitive index of this type of combination. At low polysaccharide concentrations, di-diol borate combination might reduce the mean axial ratio through partial or complete alignment of pairs of molecules. This would reduce the viscosity, but by increasing the mass and diminishing the frictional coefficient would increase the rate of sedimentation.

Mono-diol borate combination, favored by low concentrations of diol, and by low ratios of diol to borate when the diol concentration is high, appears to have occurred to the virtual exclusion of di-diol borate combination at polysaccharide concentrations of 2.5 mg./ml., regardless of borate concentration, and in 0.05 *M* sodium borate at polysaccharide concentrations of 5 and 10 mg./ml. as well. With rising borate concentration and increasing proportions of the mono-diol borate compound the sedimentation constant rose gradually from 7.1 to 8.0 *s*. In view of the following considerations, a change of this magnitude is reasonable.

Mono-diol borate combination adds $BO_2 = 42.82$ to the weight of the sugar residue (see Eq. 3). This would increase the residue weights of galactose and fucose, for example, the diol-containing sugars which have been isolated from the hydrolysis products of certain preparations of blood group substance (19), by 26.4% and 29.3%, respectively. The BO_2 group, even in its more extended tetrahedral form (20), might be expected to increase the density as well as the weight of the molecule. Furthermore, the borate groups, by increasing the width of the molecule, should reduce (as mentioned in the discussion of viscosity) both the axial ratio and the frictional coefficient. All 3 factors should operate to increase the sedimentation constant.

If the frictional coefficient and partial specific volume of a substance are known, the molecular weight can be computed from the sedimentation constant by the relation (21):

$$M = [6\ (f/f_o)\ s_{20}\pi\eta N(3V/4\ \pi N)^{\frac{1}{3}}/(1 - V\rho_{20})]^{\frac{3}{2}},$$

in which the symbols have their usual significance. Provided hydration is negligible and the molecules are randomly oriented in the viscometer, the frictional coefficient (f/f_o) can be computed from the intrinsic viscosity by means of the Simha equation (22) relating viscosity and axial ratio for an elongated ellipsoid of revolution, and the equation of Perrin (23) and of Herzog, Illig and Kudar (24) relating axial ratio and frictional coefficient. The intrinsic viscosity, 78.5, in borate-free solution corresponds to an axial ratio of 31.3 and a frictional coefficient of 2.41. Using $V = 0.7$ and $s_{20} = 7.1\ s$, the molecular weight is 278,200, the length 935 Å, and the diameter 30 Å. The reliability of these values is uncertain, however, for hydration would be expected in the neighborhood of the charged sulfate groups which the polysaccharide contains (4), and partial orientation in the viscometer has been demonstrated for molecules of even lower dissymmetry (25). Similar calculations, using the sedimentation constant and viscosity of the polysaccharide in 0.05 M sodium borate, to determine the extent of borate combination, would be even less reliable because of uncertainty concerning the effect of borate combination on the partial specific volume of the polysaccharide and on hydration.

The sedimentation and viscosity data appear to support and extend the conclusions drawn from the electrophoresis data (4) concerning the composition of the various mixtures with respect to mono-diol and di-diol borate combination. They provide further indication that the increased sharpness of the electrophoresis boundaries in the presence of borate was caused by changes in the electrical properties of the polysaccharide.

SUMMARY

At pH 8.6, sodium borate, in concentrations up to 0.05 M, progressively reduced the viscosity of dilute solutions of blood group polysaccharide and increased the sedimentation constant gradually from 7.1 to 8.0 s. In concentrations up to 0.025 M, sodium borate increased the viscosity of concentrated solutions and produced a further increase in the sedimentation constant; in 0.05 M sodium borate, on the other

hand, concentrated solutions as well as dilute solutions were reduced in viscosity, and the sedimentation constant was independent of polysaccharide concentration throughout the same range as in borate-free solution. These changes may be attributed to predominance of mono-diol borate combination at low polysaccharide concentrations, or at high polysaccharide concentrations when the ratio of polysaccharide to borate is low, and the occurrence of di-diol borate combination, which links adjacent polysaccharide chains, at high concentrations of polysaccharide when the ratio of polysaccharide to borate is high.

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The Physiological Activity of Some Simple Unsaturated Lactones. II. Effect on Certain Tissues of Higher Plants

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INTRODUCTION

The effect of certain unsaturated lactones on the growth of microorganisms and of some animal and plant tissues has been studied by a number of workers, whose results have been adequately reviewed recently by Haynes (7). Lactones such as "parasorbic acid" (D-δ-hex-α-enolactone, isolated from the fruits of *Sorbus aucuparia*) have been shown to suppress the germination of seeds—the "blastocholine" effect of Köckemann (10); and also to affect the cell-elongating properties of β-indoleacetic acid (13). Further work on similar lines, using simple synthetic lactones including DL-δ-hex-α-enolactone is reported in this communication.

EXPERIMENTAL

The lactones used (kindly supplied by Dr. L. J. Haynes and Mr. M. C. Whiting) were synthesised by the method of Haynes and Jones (8), and comprised the following: DL-δ-hex-α-enolactone; DL-δ-pent-α-enolactone; and DL-γ-hept-α-enolactone.

1. Germination of Seeds

The inhibitory effect of the 3 lactones on the germination of seeds of cress (*Lepidium sativum*) has been reported by Haynes (6) (see also 4,11,1). In the present experiments, seeds of white mustard (*Sinapis alba*) were used, and results precisely parallel to those of Haynes were observed. Hexenolactone was found to be rather more active than either of the other compounds, but in all cases an effect was observable with the lactone at a concentration of 1 in 5000 (aqueous solution), while at 1 in 1000 no sign of germination was apparent in 72 hr. in darkness at 25°C.

In view of the demonstration by Hauschka (5) that β-alanine is able partially to protect the flatworm *Dugesia tigrina* from the tissue-destroying effect of parasorbic acid, and the observation of Buston and Roy (2) that the same amino acid can counteract the effect of hexenolactone on certain microorganisms, tests were made to ascertain whether a similar protective influence could be shown in the case of seed germination.

Hexenolactone was used at a concentration of 3.5×10^{-3} *M*, and β-alanine was added over a range of concentrations from 1.2 to 5.6×10^{-3} *M*. In no case was any influence of the amino acid detected.

Similar experiments were carried out to discover whether thiamine or ascorbic acid would cause any reversal of the lactone inhibition, following the suggestion of Chakravarti (3) that the loss of germinative capacity in barley seeds exposed to ethylene oxide was connected with the destruction of these vitamins. In no case was any effect produced by thiamine or ascorbic acid, alone or in combination.

2. *Growth of Excised Roots*

The nutritional needs of excised tomato roots are well known, and cultures of these were made according to the method of White (17). Table I shows that hexenolactone at a concentration of 1 in 10,000 causes almost complete cessation of growth. Since an external supply of thiamine is necessary for the growth of tomato roots in synthetic media, hexenolactone cannot act by blocking the synthesis of this substance in the tissues; it might, however, interfere with its subsequent utilization, in which case an additional supply of thiamine might overcome the inhibition. However, experiments failed to reveal any protective influence of thiamine. Nicotinic acid also was ineffective.

TABLE I

Effect of DL-*δ-Hexenolactone on the Growth of Tomato Roots*

(Figures indicate increase in length (mm.) in 7 day peirods)

1st period	2nd period	3rd period lactone		4th period lactone		5th period lactone	
		Nil	1/10,000	Nil	1/10,000	Nil	1/10,000
20.0±1.2[a]	40.5±1.5	30.6±2.4	1.2	14.0±1.2	0.8	14.0±1.3	0.6
23.0±1.4[a]	7.5±0.2	29.5±6.4	0.3	26.5±2.0	0.6	—	—

[a] No thiamin added to basal medium; in other cases, 50 γ/25 ml.

3. *Cell Elongation* (*Auxin Effect*)

Interest has been shown by various workers in the relation between unsaturated lactones and those substances which promote cell-extension growth, collectively known as the auxins. The antogonistic effect of various plant extracts to synthetic auxins has been observed by more than one worker (14,9,13), and recently Larsen (13), using the *Avena* test, has studied directly the antoganism between β-indoleacetic acid and some simple unsaturated lactones, including parasorbic acid (D-δ-hex-α-enolactone), and has shown that this substance in concentrations from 1 in 1000 to 1 in 200 markedly affects the curvaturs of the *Avena* coleoptile caused by the auxin.

In the first experiments, use was made of the simple and rapid test using lettuce seedlings, as described by Lacey (12). This test is hardly capable of precise quantitative application, but was sufficient to demonstrate the antagonism between hexenolactone and indoleacetic and indolepropionic acids. For purposes of more accurate study, the *Avena* test, as described by Went and Thimann (16) was used. Indoleacetic acid and hexenolactone were applied to the decapitated *Avena* coleoptile, separately and together, at various concentrations. With hexenolactone alone, no curvature resulted (Larsen reported some positive curvature at 1 in 200 of parasorbic acid), but in suitable concentrations the lactone was found to inhibit the cell elongating effect of indoleacetic acid, as is shown in Table II. This is in agreement with the findings of Larsen, although this worker observed a greater relative inhibition, possibly owing to the fact that he employed the D-lactone, while in the present work the DL-form was used.

TABLE II

Effect of DL-*δ-Hexenolactone on the Activity of β-Indoleacetic Acid and of Natural Auxin Preparations in the Avena Test.*

(Figures indicate mean curvature of the *Avena* coleoptiles, in degrees)

Nature of the auxin used	Concentration of hexenolactone, 1 in							Control without lactone
	200	400	800	1,500	2,000	3,200	10,000	
β-indoleacetic acid 50 γ/l.	2.7	3.4	7.4[a]	12.7	15.1	—	16.0	19.5
Extract of *Avena* coleoptiles	—	0	0	0	—	0	—	4.2
Extract of shoot of Myrobalan plum	—	<2	<2	—	—	5.5[b]	—	>8.4

[a] Hexenolactone, 1 in 1000.

[b] Hexenolactone, 1 in 4000.

Similar tests were carried out, using preparations of natural plant auxins, in place of the synthetic substances. This was thought desirable in view of the uncertainty as to the precise role of indoleacetic acid in the normal growth of the coleoptile. Extracts containing the natural auxins were prepared from *Avena* coleoptiles by the ether method recommended by Thimann and Skoog (15). As shown in Table II this extract had a rather weak auxin activity, and its effect was completely inhibited by hexenolactone at a concentration of 1 in 3200. An extract of natural auxins was also obtained from Myrobalan B plum shoots, and again it was found that the curvatures produced by this extract on *Avena* coleoptiles were markedly reduced in the presence of hexenolactone. Experiments on the effect of the lactone on root-formation in cuttings of both soft-wooded and hard-wooded plants were inconclusive.

The protective action of β-alanine was again examined, using indoleacetic acid as the auxin. With 50 γ/l. of this substance, and in the presence of 1 in 1000 (approximately 8.5×10^{-3} *M*) hexenolactone, complete protection was afforded by β-alanine at concentrations from 2.2×10^{-3} *M* to 4.3×10^{-3} *M*. Higher concentrations afforded less protection (5) (Table III).

TABLE III

Effect of β-Alanine on the Inhibition of Activity of β-Indoleacetic Acid by DL-*δ-Hexenolactone* (*Avena Test*)

(Figures indicate mean curvature of the *Avena* coleoptiles, in degrees)

Control—50 γ IAA/l. (no lactone)	Hexenolactone, 8.5×10^{-3} *M* (1 in 1000, approx.) plus β-alanine				
	0	2.2×10^{-3} *M*	4.3×10^{-3} *M*	5.4×10^{-3} *M*	8.5×10^{-3} *M*
21.0	7.0	20.2	21.3	13.9	13.9

DISCUSSION

The results of the experiments on the germination of seeds are in general agreement with those previously published, the only new point being the failure of β-alanine to overcome the inhibitory effect of hexenolactone. This seems to be the first recorded case of such a failure, and is interesting in that it suggests that the protective effect of β-alanine against hexenolactone is not due to the formation of a physiologically inactive substance by chemical combination of the two substances outside the cells.

Root cultures resemble to some extent those of microorganisms, in that the tissue is capable of growth on a relatively simple medium, with possibly the addition of one or two growth factors; it is not surprising, therefore, that roots resemble microorganisms in their sensitivity to the lactones.

Turning to the auxin question, it has now been shown that lactone inhibition applies to the natural auxins present in *Avena* coleoptiles and in Myrobalan plum shoots, as well as to β-indoleacetic acid (13). The auxins naturally occurring in the plant are known to contain auxins *a* and *b* in addition to indoleacetic acid, although why substances of such different chemical structures exhibit similar activity is by no means clear. Until more is known concerning the chemical basis of cell elongation and auxin action, speculation as to the manner in which hexenolactone acts would seem premature, but it is quite possible that its effect depends upon some interference with the general metabolism of the plant cells (*e. g.*, respiration) and that the anti-auxin effect is secondary. The fact that β-alanine can afford some protection for the auxin may be taken in support of this view, for there has been no suggestion that this amino acid has a primary function in cell extension, though it may have some other metabolic role.

SUMMARY

1. The inhibitory effect of DL-δ-hexenolactone on the germination of mustard seeds is not overcome by β-alanine, thiamine or ascorbic acid.

2. DL-δ-Hexenolactone at a concentration of 1 in 10,000 completely inhibits the growth of excised tomato roots; the inhibition is not counteracted by thiamine or nicotinic acid.

3. It is confirmed that in the *Avena* test DL-δ-hexenolactone is antagonistic to β-indoleacetic acid. No positive curvature of the coleoptiles was observed with the lactone alone, nor did the lactone itself show any auxin activity at low concentrations.

4. In the same test, the lactone was found to be antagonistic to plant extracts containing the natural auxins.

5. The antagonism of hexenolactone to β-indoleacetic acid in the *Avena* test is overcome by certain concentrations of β-alanine.

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On the Mechanism of Enzyme Action. XXXVI. Dehydrogenation Studies on *Merulius lacrymans* and *Marasmius chordalis*, and the Mechanism of Oxalic Acid Formation

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INTRODUCTION

It may be safely assumed that molds in their natural habitat become adapted to growth under threshold nutritional conditions. It is necessary, therefore, to account for appreciable amounts of their metabolic products, to cultivate such organisms in media containing carbohydrates in concentrations which are excessive as compared with those found in their natural conditions. In agreement with Kluyver and Perquin (1), it may be possible under such "pathological" conditions to obtain a chemically interpretable picture of their enzymological activities.

In continuing the studies conducted in this laboratory and leading to an attempt at a better understanding of the pathways of lignification, two organisms, which may be regarded as amplifying each other in regard to their enzymic efficiency, were selected at the suggestion of Dr. Nord. Both are members of the class *Basidiomycetes*. One, *Merulius lacrymans,* is a typical wood destroyer, while the other, *Marasmius chordalis,* is a soil inhabitant. *Merulius lacrymans* (Melac) has been under investigation for some years because of its relation to the destruction of domestic timber (2), and the effects observed indicated that wood cellulose is almost completely destroyed in advanced stages of decay by this mold, but that the lignin present is altered to a substance resembling humic acid (3). The group of *Marasmii* has been studied in Sweden from the physiological viewpoint (4), using sterile pine needles as substrate material, to determine the effect of molds of this type on lignin and cellulose. In his investigation, Lindeberg reported finding that one member of the group, *Marasmius chordalis* (Machor), which in nature decomposes straw and litter to humus, was an excellent cellulose destroyer but did not attack lignin. Thus, both molds in nature primarily possess the function of decomposing of cellulose, but may be considered as different in regard to their enzymatic capacity for attacking lignin.

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The microanalyses were carried out by M. Bier of this Department.

In the present work, using various carbohydrates and alcohols as carbon sources, we have followed the intermediate metabolism of such substances with these organisms. By means of periodic chemical analyses, mycelial weights were determined, as well as the residual sugar, volatile acids, alcohol, and oxalate formed, together with the pH of the filtered medium. By plotting the results of such analyses we can follow the trend of the metabolic processes which express themselves in the final balance of products.

By incorporation of the dye resazurin in the culture medium (5) and its subsequent discoloration, it was indicated that both these molds possessed strong dehydrogenating systems. Melac caused more rapid discoloration of the resazurin added to the culture medium than Machor, but, for the purpose of the enzyme studies, pregrown mats of the latter often proved more useful.

CULTURES AND MEDIA

The stock cultures of the molds have been maintained on a nutrient agar, made of the same components as the liquid glucose medium (see below), supplemented by 20 g. of Difco Bacto-Agar/l., 50 ml. of this mixture being used as a plate/125 ml. Erlenmeyer flask. The flasks were cotton plugged and inoculated by transferring mycelium by means of a platinum loop. Flasks were kept in the dark during all growth periods. Most series were kept in a low-temperature incubator at 22°C. In special cases, where noted, growth was at room temperature, ranging up to 28°C.

For metabolism studies, a plate of about one month's growth was used for preparing the spore-mycelial suspension used for inoculation, 50 ml. of sterile distilled water being poured into the flask, and the mycelium loosened from the agar base by gently scraping with a platinum loop. One ml. of this suspension was used to inoculate 50 ml. of medium, the transfer being made by means of a sterile syringe.

In dehydrogenation studies, transfers of pregrown mat sections or whole mats from regular glucose media were used, applying aseptic technique. Such mats were rinsed in sterile distilled water several times before transfer. Glucose analyses on such rinses, and on media to which such pregrown mats have been transferred, indicate that this method removes all but negligible traces of the glucose, the latter being equivalent to not more than 0.4 mg. Cu_2O in the Munson-Walker determination.

Where a carbon source to be used was thought likely to be toxic to the mold at higher concentrations, as with isopropyl alcohol, a preliminary trial was made to determine the optimal concentration for growth. By such a comparison, 0.4% isopropyl alcohol was found to offer the best concentration for growth of Melac.

Large scale experiments, set up for the purpose of isolating specific substances, were prepared in 3 l. Fernbach flasks, 1 l. of medium being used per flask, and inoculations were made with a mat of the mold which had been grown on the same medium.

The medium used in this work consists of a carbon source, for example:

Glucose[2]	20 g.
(or other carbon source as noted in the text)	

supplemented by

KH_2PO_4	1.5 g.
Neopeptone (Difco)	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Thiamine hydrochloride	.2 mg.

and made up to 1 l. with tap water. Fifty ml. of this medium was used in each 125 ml. Erlenmeyer flask.

Analyses were run on the combined contents of two flasks, all analyses being carried out in duplicate. In all growth series of 20 flasks, at least two were uninoculated to serve as blanks for that series. In cases in which discoloration of resazurin was observed as evidence of dehydrogenation, a concentration of 1.5×10^{-5} *M* was used in two flasks prior to sterilization. One of the latter was inoculated, the other kept as a blank.

The *Merulius lacrymans* strain was obtained through the courtesy of Dr. Wm. J. Robbins of the New York Botanical Gardens, New York 58, N. Y., the culture of *Marasmius chordalis* through the courtesy of Prof. E. Melin, Dept. of Physiological Botany, Royal University, Uppsala, Sweden.

The adonitol used in this study was a gift from Dr. H. M. Wuest of Hoffmann-La-Roche, Inc., Nutley, N. J.

ANALYTICAL METHODS

Mycelial weights were determined by filtering off the medium through a tared alundum crucible, washing the mycelium with distilled water, drying in an oven at 60°C., and weighing.

pH of the medium was determined by means of a Cambridge electron-ray pH meter.

Glucose has been determined as described in the Munson-Walker method (6) in all cases. However, where a corrected value is needed, due to the presence of interfering substances, the method developed for determining true glucose in blood (7) has been adapted to this work. This preliminary treatment with $HgSO_4$ reagent has proven satisfactory for use on samples of media.

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[2] The glucose used here is Cerelose of the Corn Products Ref. Co.

Oxalate has been determined by the method described by Bernhauer (8), alcohol by the method of Janke and Kropacsy (9), following a preliminary treatment to remove aldehydic substances (10). The alcohol produced in these experiments is identified as ethyl alcohol, since dichromate oxidation to acid, followed by steam distillation of the acid so produced, and determination of the partition coefficient (11), shows conversion to acetic acid.

The volatile acid produced in these experiments has been ascertained to be acetic acid by steam distillation, followed by determination of the partition coefficient (11). Titration of an aliquot of the steam distillate with NaOH gave the quantitative relationship.

Acetone has been determined by the iodoform method (12).

Results

Growth of Melac on a 2% glucose medium at 22°C., the known optimal temperature as shown in Fig. 1, results in oxalic acid as the main isolable end-product of metabolism, reaching a maximum of 98 mg./100 ml. on a glucose medium.

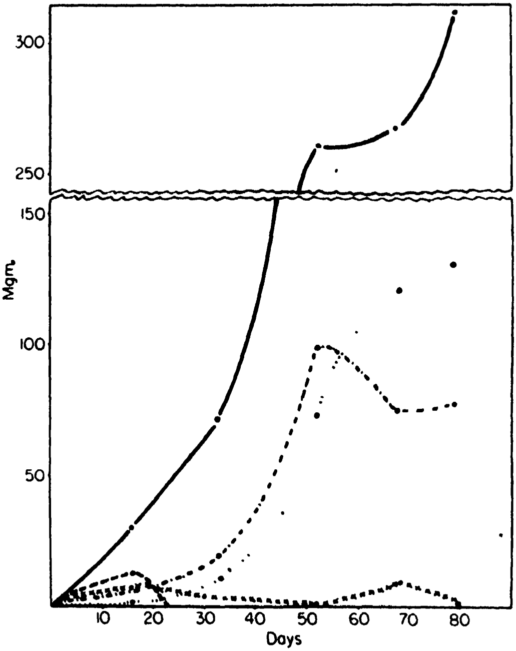


Fig. 1. Growth of *Merulius lacrymans* on 2% glucose medium, at 22°C.

●————● Mycelial wt., mg./100 ml. ●–·–·–·● Oxalate, mg./100 ml.

●········● Glucose utilized, mg./10 ml. ●– – – –● Acetate, mg./100 ml.

●××××● Alcohol, mg./100 ml.

Ether extraction of a filtered Melac medium in a liquid-liquid extractor, with subsequent evaporation of the ether, left a brown syrup, which gave on vacuum sublimation a colorless crystalline compound of m.p. 189°C., and a neutral equivalent of 45, corresponding to anhydrous oxalic acid. Furthermore, a calcium salt with 2 moles of water was obtained by addition of excess 10% $CaCl_2$ solution, to the cold medium. This precipitated after standing over night in the refrigerator, giving, after recrystallization (13),

Calculated: C, 14.64%; H, 2.44%; found: C, 14.50%; H, 2.49%

corresponding to $Ca(C_2O_4) \cdot 2H_2O$.

In general, it may be said that oxalate production parallels the disappearance of glucose from the medium, and the increase in mycelial weight. Acetic acid is shown to be produced in small amounts, up to 13 mg./100 ml. in this series, and ethyl alcohol up to 9 mg./100 ml.; both substances appear, however, only during periods of slow growth, when their utilization is slowed down and they are allowed to accumulate, that is, at the latent phase of early growth, or at the later stationary phase. The pH of the medium drops during growth from 5.6 to 2.6.

The temperature maximum for Melac is 25–26°C., and it is extremely sensitive to temperature variations. A glucose series, similar to the above in all respects, but grown at room temperature, shows noteworthy differences in the growth and product curves (Fig. 2). In the early part of this series, growth temperatures were low, 22–25°C., and growth proceeded at about optimal rate. In the latter half of the growth period, however, the room temperature had risen, and at intervals exceeded 27°C. Here the growth curve may be seen to level off, despite the fact that an ample supply of glucose still remained in the medium. In this series, acetic acid also appeared but accumulated to a higher level (40 mg./100 ml.), due to the slow rate of metabolism. Oxalate again followed the mycelial weight curve, reaching the maximum of 121 mg./100 ml.; however, a drop in level occurred coincident to acetic acid accumulation, indicating the inverse relation in the balance of these products. The pH in this series dropped from 5.5 at the onset of growth to 3.3.

The utilization of glucose, followed, as before, the formation of mycelium until the 31st day of growth, when the apparent glucose content of the medium leveled off, despite the continued development of mycelium. Prolongation of this trend to the 38th day, and subsequent analyses, led to the assumption that a product of aldehydic nature must be causing interference in the Munson-Walker analyses. The samples were then treated with $HgSO_4$ reagent to remove the interfering substance, and they now showed the expected glucose levels. The Cu_2O in the untreated specimen in some cases amounted to as much as 20% in excess of the treated sample.

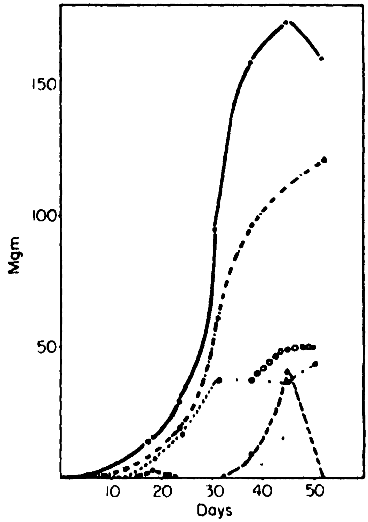


FIG. 2. Growth of *Merulius lacrymans* on 2% glucose medium, at room temperature, ranging up to 28°C.

●————● Mycelial wt., mg./100 ml.
●········● Glucose utilized, uncorrected, mg./10 ml.
●○○○○● Glucose utilized, corrected, mg./10 ml.
●–·–·–·–● Oxalate, mg./100 ml.
●– – – –● Acetate, mg./100 ml.
●××××● Alcohol, mg./100 ml.

Addition of phenylhydrazine and a trace of acetic acid to the medium in the cold, resulted in an immèdiate formation of lustrous white platelets, a characteristic test for glyoxylic acid. The melting point of this derivative, 147°C., corresponded with that given in the literature, and a mixed melting point with known glyoxylic acid phenylhydrazone showed no depression. Nevertheless, the difficulty experienced in obtaining a sample of analytical purity was in agreement with previous observations (14). In addition the *p*-nitrophenylhydrazone, m.p. 200°C., and the 2,4-dinitrophenylhydrazone, m.p. 189°C., were also prepared from a sample of media, both melting points corresponding with those found in the literature.

Quantitative calculations have been made of the amount of glyoxylic acid corresponding to the yield of the crude phenylhydrazone isolated from large-scale (1 l.) regular glucose media on which Melac has been grown at room temperature. Such data show that 0.63 and 1.06 g. of glyoxylic acid were formed per l. in 4- and 5-month samples of the same series. Simultaneously with the latter, treatment of a 2-month sample of a similar series showed no glyoxylic acid to be present.

A convenient demonstration of the dehydrogenating enzyme system in Melac is shown by transfer of a small mat section (about 20 mg.) to a medium containing 0.4% isopropyl alcohol as carbon source (15). On such a medium, resazurin is rapidly decolorized, and iodoform analysis of a 27 day old sample with 35.2 mg. of mycelium (grown at room temperature) and of a 19 day old sample with 76 mg. of mycelium (grown at 22°C.) shows the presence of 6.6 and 10.4 mg. of acetone/100 ml. of medium, respectively.

To isolate a derivative, a 100 ml. sample of filtered medium was distilled into a saturated solution of 2,4-dinitrophenylhydrazine in 1 *N* HCl, and a precipitate formed immediately in the receiving flask. The yellow crystals, after washing and vacuum drying, weighed 31.5 mg. and had a melting point of 126°C. A sample (recrystallized) proved on microanalysis to be acetone 2,4-dinitrophenylhydrazone, $C_9H_{10}O_4N_4$.

Calculated: C, 45.37%; H, 4.20%; found: C, 45.47%; H, 4.02%.

Calculation shows that the derivative weight corresponds to 7.7 mg. of acetone/100 ml. of medium. The correspondence of analysis and derivative results all indicate that the conversion of isopropyl alcohol to acetone is 2–3%.

Interval analyses of the isopropyl alcohol medium also indicate that oxalate, acetate and traces of an alcohol are formed from this carbon source.

With Machor the main product to accumulate on a 2% glucose medium is ethyl alcohol; in fact, its presence may be detected by the odor while growing. In general, as in the typical case illustrated by Fig. 3, *ca.* 50 mg./100 ml. is the maximum alcohol level attained; however, in similar cultures as much as 80 mg./100 ml. has been reached. The alcohol formed accumulates during the early slower growth period, reaching its maximum at about the 40th to the 60th day. When rapid growth begins, the alcohol is dehydrogenated and acetic acid and oxalate appear, acetic acid reaching a level of *ca.* 18 mg./100 ml. maximum,

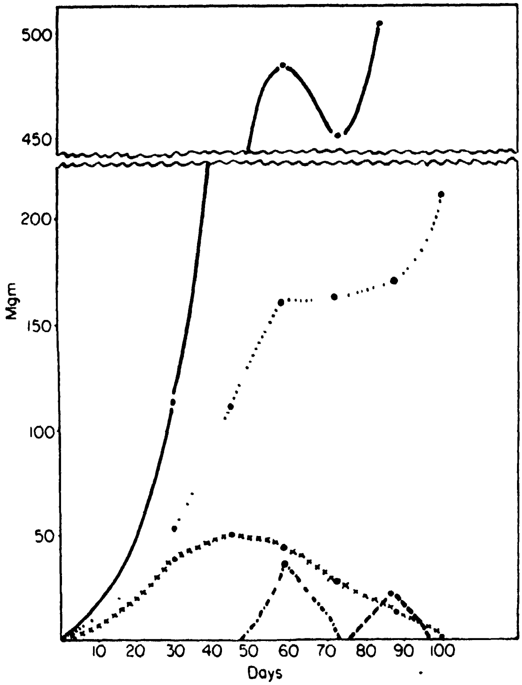


FIG. 3. Growth of *Marasmius chordalis* on 2% glucose medium, at 22°C.

● ——— ● Mycelial wt., mg./100 ml. ●-·-·-·-● Oxalate, mg./100 ml.
●·······● Glucose utilized, mg./10 ml. ●— — — —● Acetate, mg./100 ml.
●×××××● Alcohol, mg./100 ml.

and oxalate 36 mg./100 ml. maximum. Here again, as with Melac, we observed an inverse quantitative relationship between alcohol and its dehydrogenation products, acetate and oxalate.

On a 2% sorbitol medium, using a pregrown mat transfer, the metabolism was, as recorded in Table I, very similar to that on a glucose

TABLE I

Growth of Marasmius chordalis (Mat Transfer) on 2% Sorbitol Medium

Growth period	Mycelial weight	Reducing sugar	Oxalate	Alcohol	Acetate	pH
days	*mg./100 ml.*		*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	
4	653	+	None	13.2	None	5.16
20	678	Trace	5.9	21.8	None	5.66

medium, resazurin being decolorized, and reducing sugar, as well as quantities of ethyl alcohol and oxalate, being formed. Furthermore, in some similar series on this medium, acetate was also detected.

Another higher alcohol studied with the aid of a pregrown mat transfer, was a 1% adonitol medium. As recorded in Table II the detection of ethyl alcohol as well as oxalate is evidenced by the data.

TABLE II

Growth of Marasmius chordalis (Mat Transfer) on 1% Adonitol Medium

Growth period	Mycelial weight	Reducing sugar	Oxalate	Alcohol	Acetate	pH
days	*mg./100 ml.*		*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	
4	484	Trace	None	18.8	None	4.64
20	526	Trace	None	27.2	None	5.05
33	534	Trace	7.1	1.6	None	5.98

Using a spore-mycelial inoculation on 0.5 and 1% ethyl alcohol as carbon source (Table III), Machor has been shown to produce small amounts of oxalate and acetate, which are, however, rapidly consumed as soon as the alcohol level is measurably lowered.

TABLE III

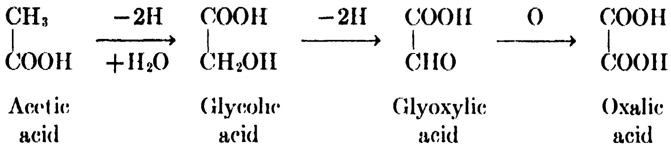
Growth of Marasmius chordalis on Ethyl Alcohol Medium, Spore-Mycelial Inoculation

Series I—1% ethyl alcohol; Series II—0.5% ethyl alcohol.

Growth period	Mycelial weight		Alcohol remaining		Oxalate		Acetate		pH	
	I	II	I	II	I	II	I	II	I	II
days	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg /100 ml.*		
0	0	0	960	480	0	0	0	0	5.23	5.63
49	22	30	169	55	None	None	7.46	None	5.97	5.91
63	50	52	88	19	4.83	11.9	None	None	5.71	5.61
77	78	80	41	8	None	None	None	None	5.48	5.88
92	110	75	1	None	None	None	None	None	—	—

DISCUSSION

Working with molds of the wood destroying group, Nord and Vitucci (16) postulated the phase sequence



$$\begin{array}{c} CH_3 \\ | \\ COOH \end{array} \xrightarrow[+H_2O]{-2H} \begin{array}{c} COOH \\ | \\ CH_2OH \end{array} \xrightarrow{-2H} \begin{array}{c} COOH \\ | \\ CHO \end{array} \xrightarrow{O} \begin{array}{c} COOH \\ | \\ COOH \end{array}$$

as one of two alternate mechanisms of acetate metabolism. These workers, using several species of the *Merulii* and *Fomes annosus*, demonstrated the formation of oxalate from acetate and glycolate. Using the discoloration of resazurin as an indication of a dehydrogenation step, and showing the formation of oxalate from glycolate, they postulated the intermediate formation of glyoxylic acid. The present work has furnished the experimental evidence for the formation of this acid in appreciable quantities on the pathway from glucose to oxalic acid by Melac.

Thus, the postulation of the importance of the sequence above as one of the major routes of carbon source utilization by molds is corroborated by the following facts noted in this study:.(1) The finding of appreciable quantities of glyoxylic acid, in addition to acetate and oxalate, formed by Melac from glucose: (2) The demonstration of the dehydrogenating ability of this organism by the formation of acetone from isopropyl alcohol, an alcohol not natural to this mold; (3) The accumulation of ethyl alcohol, the predecessor of the above sequence by Machor, as well as the production of acetate and oxalate from glucose, and also from the C_6 alcohol, sorbitol, and the C_5 alcohol, adonitol: (4) The ability of Machor to convert ethyl alcohol into acetate and oxalate.

It is interesting to insert these observations into the general picture of carbohydrate degradation by molds. Dakin (14) stated that glyoxylic acid is frequently detected in cultures of molds and bacteria, but gave no details. Raistrick and Clark (17) found scarcely any oxalate formed when *Aspergillus niger* was grown on glyoxylic or glycolic acid, whereas Challenger, Subramaniam and Walker (18) believe that the above mechanism holds for *Aspergillus*, since they could obtain these products in small yields from acetate. Clutterbuck suggested, in analogy to the animal, that a direct oxidation of acetic acid to glyoxylic takes place, and the latter, by a dismutation, yields oxalic and glycolic acids (18). The recent work of the Lynens (19) attempts to indicate that *Aspergillus niger* does not utilize glycolate to form oxalate, and they *argue* that glycolate, when found in certain media, may be formed reductively from oxalate, since, *in vitro*, oxalate is easily reduced, in distinction to other carboxylic acids.

Significant evidence, however, for the intermediary occurrence of glycolic acid in the formation of oxalic acid by molds, rests in the *experimental* fact that Chrzaszcz *et al.* (20,21), and Nord and Vitucci (*l.c.*), also obtained this substance from pyruvic as well as lactic acid. This observation seems, therefore, to invalidate the reasoning of the Lynens in their discussion with A. Allsopp (22) *vs.* the availibility of glycolic acid in the transition of acetic acid to oxalic acid in the case of pregrown mats of *Aspergillus niger.*

Furthermore, there are indications that glycolic and glyoxylic acids play a role in the metabolism of fungi (23) and higher plants (24a,b,c). From yeast maceration juice containing added glyoxylic acid, at pH 5-6, glycolaldehyde, glycolic, oxalic and other organic acids were formed. Here oxalic and glycolic acids were produced in equimolar quantities from the glyoxylic acid by oxidation-reduction.

It is also of interest that the work of Wehmer (25), which was concerned with oxalate formation by *Aspergillus niger* in particular, and to some extent with *Penicillium glaucum,* indicated that acid conditions do not allow accumulation of oxalate in mold fermentations. In our work, oxalate production is found, even though the pH of the medium is always on the acid side, the original medium having a pH of about 5.4, which, during growth of Melac, drops to about pH 2.0. Calculations indicate that the acidity of the medium can be accounted for by oxalic acid. Even at the lower pH, oxalate production continues to parallel mycelial growth; however, the free acid can be utilized, as is shown in periods of rapid growth.

It might be, however, that deviations in utilization of varying carbohydrate sources could be accounted for by differences of the strain in the hands of the various workers, by spontaneous mutations, and by the differing conditions of their experiments. With our organisms the accumulation of a product characteristic of the particular organism, as alcohol with Machor and oxalate with Melac, appears to be governed by the rate of utilization of the intermediate products of metabolism, rather than a real difference in the mechanism of breakdown, a view supported by the fact that glyoxylic acid accumulated when the temperature conditions were not optimal.

SUMMARY

1. Work with *Merulius lacrymans* and *Marasmius chordalis* supports the course of degradation:

Acetate → Glycolate → Glyoxylate → Oxalate

as one of the pathways of the phase sequence of carbohydrate breakdown by organisms of this group.

2. Dehydrogenation of the following alcohols has been demonstrated by these molds: sorbitol, adonitol, isopropyl alcohol, and ethyl alcohol.

3. With *Merulius lacrymans*, temperature variations strongly influence the isolability of intermediates.

4. Oxalic acid production is continued by *Merulius lacrymans* even under acid conditions, the free acid accounting for the acidity of the medium.

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On the Mechanism of Enzyme Action. XXXVII.
Solanione, a Pigment from *Fusarium Solani* D_2 Purple

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Introduction

A large number of bacteria and molds produce pigments which were formerly thought of as waste metabolic products. However, as far back as 1909 it was observed by v. Tappeiner (1) that the pigment hypericin could exert a photodynamic effect on microorganisms. Since then it has been shown that these colored substances are capable, for example, of influencing the rate of dehydrogenation by molds (2,3,4), of inhibiting the action of certain enzymes (5,6), and of having antibiotic activity. It was with this experience in mind that a study was made of the structure of one of the pigments produced by *Fusarium solani* D_2 purple (FsD).

Following a preliminary report (7), a detailed description of the isolation, identification, and attempted synthesis of this pigment (solanione) will now be given.

Conditions for Growth and Isolation of Solanione

This particular species, as it grows, not only retains the pigments within the mat or mycelium, but also excretes them into the surrounding medium which changes color from orange, after 7 days of growth, to a deep purple after 4 weeks. Originally, the mold was grown on Difco potato dextrose-agar, but growth was extremely slow and the use of a liquid medium was, therefore, attempted. It was previously observed (8,9) that, when a modified Czapek-Dox medium was employed, FsD produced pigments as indicated by the deep coloration in the solution.

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[1] Communication No. 175. Presented before the Division of Biological Chemistry, Am. Chem. Soc., Washington, D. C., August, 1948. These investigations were assisted by a grant from the Office of Naval Research. The analyses presented were carried out by M. Bier of this laboratory.

To find the optimum conditions for pigment formation, a study was made of the effect of pH, temperature, and zinc ions on the latter, using 2 different media (Raulin-Thom and Czapek-Dox).

Fig. 1 presents the results of varying the pH on pigment production. It can be seen that the Czapek-Dox medium is much superior to that of Raulin-Thom, the optimum pH of the former being about 4. (The ordinate units—colorimeter readings—are arbitrary, since, at this time, no pure pigment had been isolated.)

Separate experiments at different temperatures in the presence and absence of light, carried out with both solid and liquid media, revealed that the best results were obtained between 25–30°C., and that light exerted no appreciable effect on pigment formation. Similarly, it was found that addition of zinc ions (2 mg./l.) to the Czapek-Dox medium increased pigment production, but that higher quantities tended to have a decreasing effect.

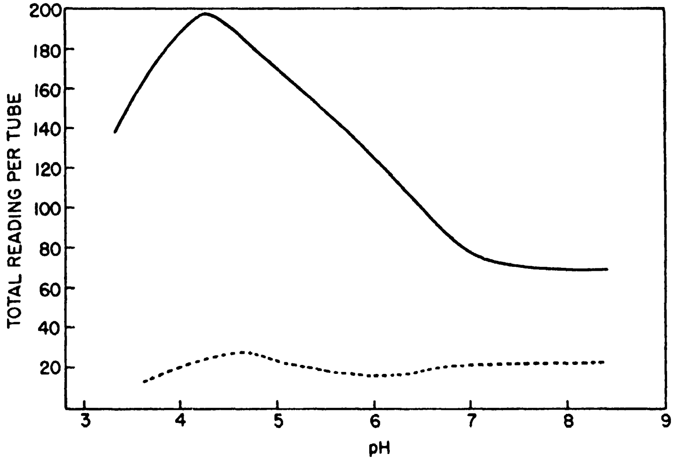


FIG. 1. Determination of optimum pH for the growth of FsD. ——— - Czapek-Dox medium. ----------- Raulin-Thom medium.

Later experience showed that as the mold grows, the pH of the solution increases and, therefore, the color change described previously (orange to purple) is due to the formation of the soluble colored salts of the pigments.

Thus, utilizing the above information, a large scale experiment was started in which FsD was grown on a modified Czapek-Dox medium for one month. The mycelia were then separated from the purple solution by filtration, the latter acidified to pH 3 with H_2SO_4 and extracted in liquid-liquid extractors for 48 hr., using ether as the solvent. Evaporation of the ethereal solution yielded an orange-red residue (m.p. 140–165°C.) which gave an intense violet color when treated with alkali; but attempts at fractionating the mixture by such methods as partition between solvents, chromatography or fractional precipitation failed as evidenced by the melting points of the various fractions. As a consequence of these results, attention was now directed toward the use of the colored mycelia as the source for the isolation of a pure pigment, and it was found that extraction of the ground mycelia with petroleum ether (b.p. 30–60°C.) for one week in a Soxhlet gave a crude orange-red product (m.p. 195–200°C.). This impure material could be recrystallized from either alcohol, ether, acetone, or benzene-petroleum ether to give an orange to reddish compound having a melting point of 208–210°C. (depending on the rate of heating).

Mention should be made at this time of the fact that FsD produces several pigments which are present in both solution and mycelia. Thus, the latter, after being exhaustively extracted with petroleum ether still retained its red color, and if either benzene, ether, or chloroform is then used as the solvent, further quantities of pigments are obtained. Apparently, however, these are mixtures and not a single entity although they show, like solanione, the characteristic color change in acid and alkali (orange to violet). There is also produced, in extremely small quantities, an orange pigment which not only is unaffected by changes in pH but is insoluble in alkali (unlike the others previously described).

Identification of Solanione

Solanione is optically inactive and contains only carbon, hydrogen and oxygen; the empirical formula derived from the quantitative data being represented best by $C_{15}H_{14}O_6$. One of the oxygen atoms occurs as a methoxyl group, while its solubility in alkali but not in $NaHCO_3$ solution suggested that at least one other oxygen atom was present as a phenolic group. This was confirmed by the violet color given with alcoholic $FeCl_3$ and the formation of a blue-violet monopotassium salt which titrated as a weak acid. Also, on acetylation with acetic anhydride and H_2SO_4, a yellow monoacetate was formed (m.p. 252–253°C.). However, during its formation, one molecule of water was lost as shown by analysis of the compound. This splitting off of water will be discussed later.

On treatment with reducing agents, such as Zn and acetic acid or sodium hydrosulfite, the orange color of the pigment is discharged to a faint yellow, which, on standing in air, slowly returned. This suggested that a quinone ring may be the chromophoric part of the molecule. Final proof for this contention was the formation of a colorless triacetate (m.p. 262–263°C.) by a reductive acetylation on the monoacetate, indicating that there were two carbonyl groups (responsible for the color of the pigment) which were first reduced to hydroxyl groups and then acetylated.

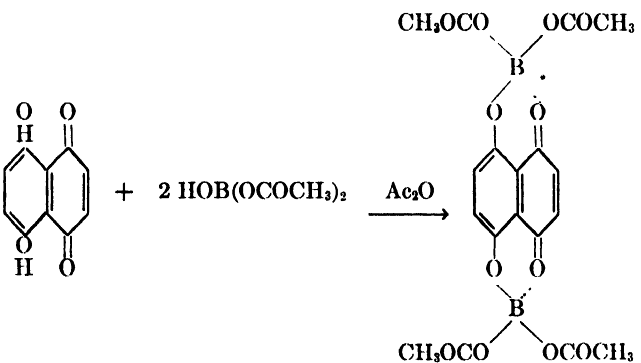
Solanione also contains a carbonyl group in addition to the quinone grouping as shown by the formation of a mono-2,4-dinitrophenylhydrazone. (Highly-substituted quinones do not react under ordinary conditions with carbonyl reagents.) This was also substantiated by the data on the infrared spectra of both solanione and the demethylated compound (Fig. 6) in which there is to be noted at about 5.80 μ the characteristic absorption of the carbonyl group (11). Other evidence, such as the reaction with sodium hypoiodite to yield iodoform, the formation of the anhydromonoacetate (with the cleavage of water), and the oxidation of solanione under certain specific conditions (Kuhn-Roth procedure) indicated that the carbonyl function was derived from a methyl ketone group.

It is known (10) that the presence of alkyl substituents can be detected by the use of a CrO_3-H_2SO_4 mixture which usually destroys the main nucleus but oxidizes any saturated parts to the corresponding acids. When solanione is treated in this manner, two moles of acetic acid were produced per mole of pigment oxidized, indicating that there are two $CH_3-\underset{\|}{C}-$ groupings in the molecule. One of these arises from the presence of a methyl ketone group and the other from a methyl to carbon (CH_3-C-) linkage.

Solanione contains 15 carbon atoms of which at least 4 are outside the main nucleus (one as a methoxy, one as a methyl, and two as methyl ketone groups). Thus, solanione is either a substituted quinone or naphthoquinone. Both chemical and physical data favored the latter.

Evidence for Type of nucleus

1. *Chemical.* Dimroth (12) has found, and later other investigators (13) have confirmed, that hydroxynaphthoquinones in which the hydroxyl group is in the *peri* position with respect to the carbonyl group, react with pyroboroacetate to form a complex addition product. *e. g.*,



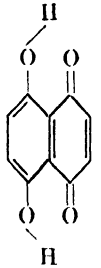
Naphthazarin Pyroboroacetate

SCHEME I

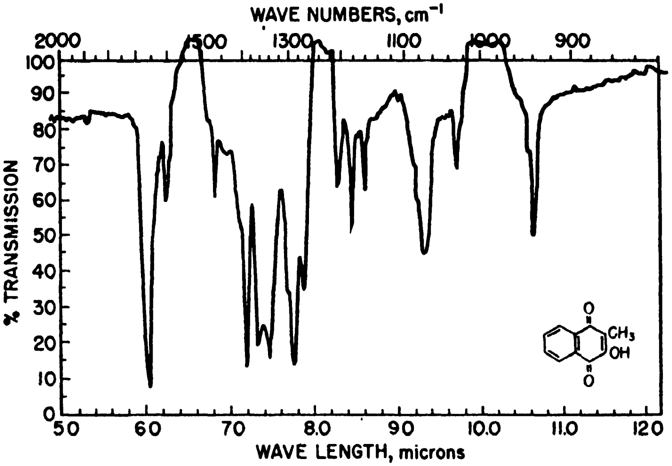
If the hydroxyl group is in any position but the *peri*, no reaction occurs; *e. g.*, 2-hydroxy-1,4-naphthoquinone fails to yield any product. Solanione reacted slowly with this reagent to form a diboroacetate derivative, signifying that it contained an α-naphthoquinone structure with two hydroxyl groups in the *peri* position. There is strong supporting evidence for this naphthazarin-type nucleus by the comparison of the infrared and ultraviolet spectra of various naphthoquinones with that of solanione or the demethylated compound.

2. Infrared Data. In Figs. 2–6 are presented the infrared absorption curves from 5 to 12 μ of different synthetic naphthoquinones as well as solanione and several of its reaction products.

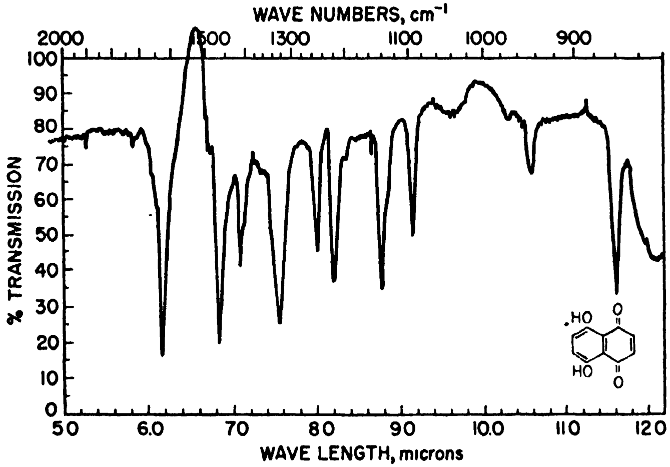
A study of the infrared spectra of quinone, naphthoquinone, and 2-methylnaphthoquinone indicates that the quinone type carbonyl vibrations are thought to occur at 6.03 μ (14). This strong absorption band is also shown in phthiocol (Fig. 2), but in naphthoquinones possessing two hydroxyl groups in the *peri* position (*e. g.*, naphthazarin, methylnaphthazarin, hydroxydreserone, and the alkylated hydroxydreserones), this band is still present but is apparently partially suppressed, as shown by the decrease in absorption at this wavelength. This is probably due to the known hydrogen bonding which exists between the hydroxyl and carbonyl groups: *e. g.*,



That such a bonding exists in these compounds is shown by the fact that the characteristic absorption band at about 2.80 μ (not shown in the curves) due to the linear vibrations of the O—H linkage is absent, in agreement with the results obtained by several investigators (11, 15,16,17) for this kind of chelation. Whether the hydroxyl absorption band was shifted to a longer wavelength as usually found for such types of compounds was not determined. It should be mentioned that, while naphthazarin and 2-methylnaphthazarin show no absorption in this region, the other naphthoquinones, such as hydroxydreserone which also contain an unbonded hydroxyl group have the expected band between 2.78–2.81 μ.



Phthiocol

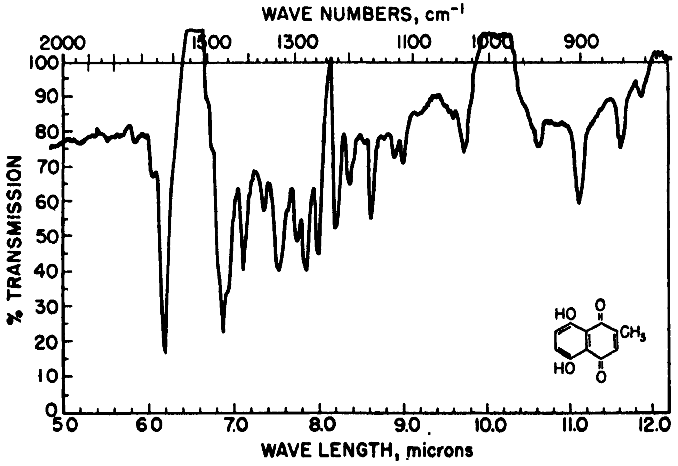


Naphthazarin

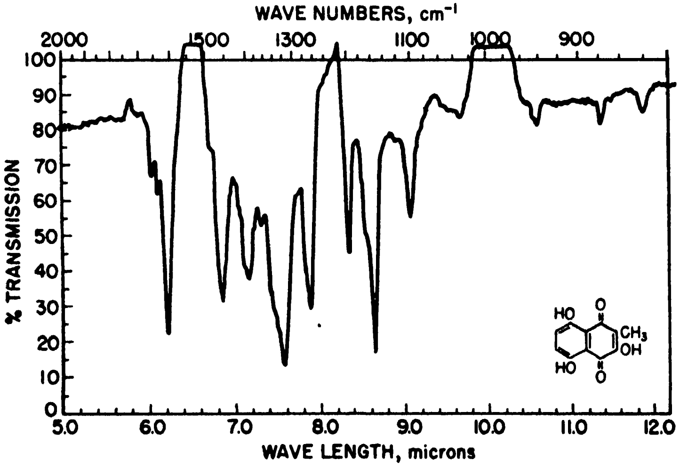
Fig. 2.

The infrared curves of solanione or the demethylated compound show the same characteristic suppression of the quinone carbonyl vibrations at 6.03 μ (Fig. 6); with the former there is no absorption at 2.80 μ (showing the absence of any free unbonded hydroxyl groups), while, with the latter, a band at 2.78 μ is present. Thus, the infrared spectral data support the contention that the pigment is a substituted naphthazarin molecule.

3. Ultraviolet Data. The ultraviolet absorption curves of solanione and the anhydromonoacetate are presented in Fig. 7.



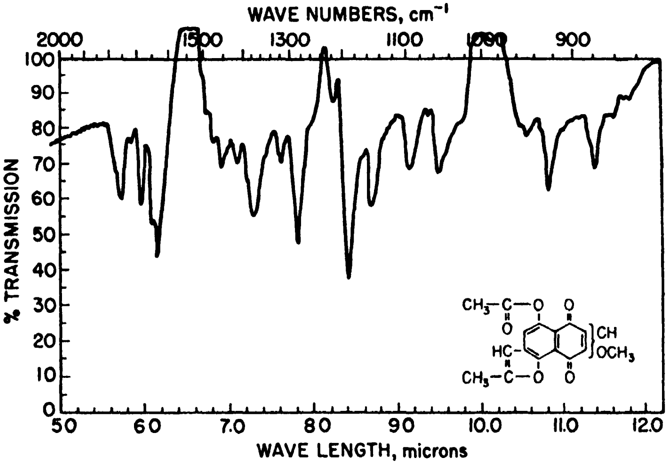
Methylnaphthazarin



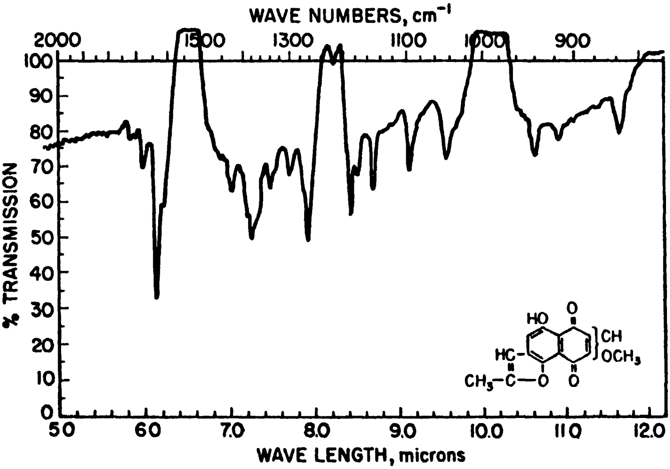
Hydroxydreserone

FIG. 3.

Macbeth *et al.* (18) concluded from the spectra of a series of different naphthoquinones, that all these compounds, with the exception of those having hydroxyl groups in the *peri* position, showed a maximum very close to 330 mμ. With the latter, either there is no maximum absorption in this region or else a point of inflection occurs. From the absorption curve of solanione, it can be seen that no maximum exists



Anhydromonoacetate

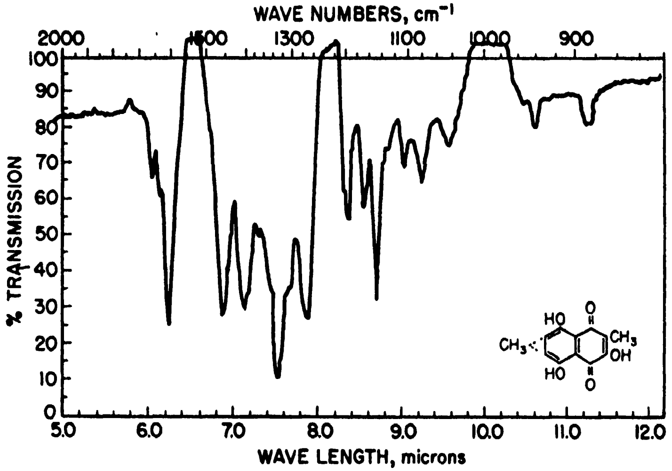


Anhydrosolanione

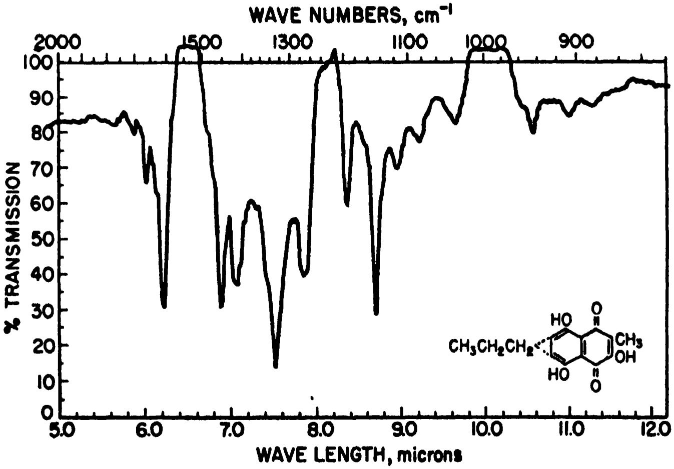
Fig. 4.

near 330 mμ which indicated that it belonged to those naphthoquinones containing hydroxyl groups in the *peri* position.

These investigators also showed that, when hydroxynaphthoquinones are acetylated, the absorption curves of the acetate are very



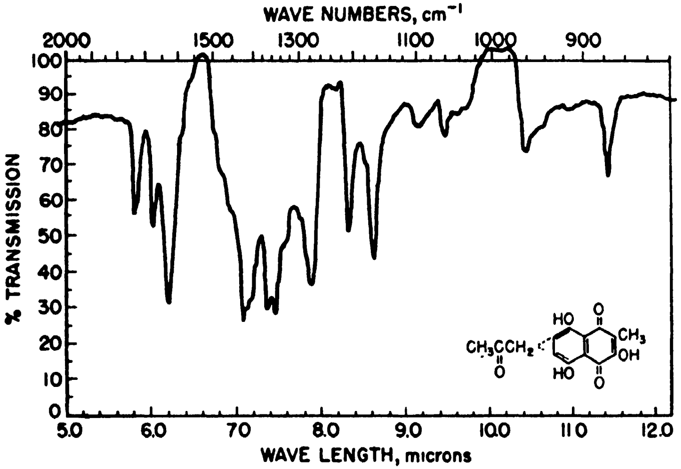
2,6 or 7 dimethyl, 3,5,8, trihydroxy, 1,4, naphthoquinone (compound III)



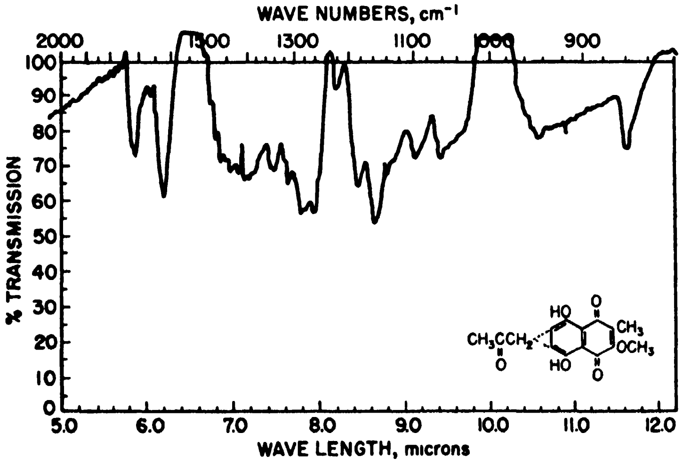
2-methyl, 3,5,8, trihydroxy, 6 or 7 propyl, 1,4, naphthoquinone (compound II)

FIG. 5. Upper: 2,6 (or 7)-Dimethyl-3,5,8-trihydroxy-1,4-naphthoquinone (Compound III). Lower: 2-Methyl-3,5,8-trihydroxy-6 (or 7)-propyl-1,4-naphthoquinone (Compound II).

similar to that of the parent compound (α-naphthoquinone). In the case of the spectrum of the anhydromonoacetate (Fig. 7), this is not quite true, since neither the number of maxima (three), nor their



Demethoxylated Solanione

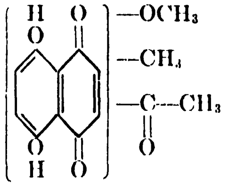


Solanione

Fig. 6.

positions, are similar to that of 1,4-naphthoquinone. This is probably due to the fact that, with this acetate, a furan ring is present in addition to the naphthalene nucleus.

Summarizing the information obtained, solanione can be represented as:



This leaves one carbon unaccounted for which is probably present as a —CH$_2$— group attached to the methyl ketone moiety. (It cannot exist as a separate methyl group for this would mean that 3 moles of acetic acid should be produced by the Kuhn-Roth procedure whereas only 2 were found.) This acetonyl grouping (CH$_3$COCH$_2$—) assists in explaining the splitting off of water during the formation of the monoacetate.

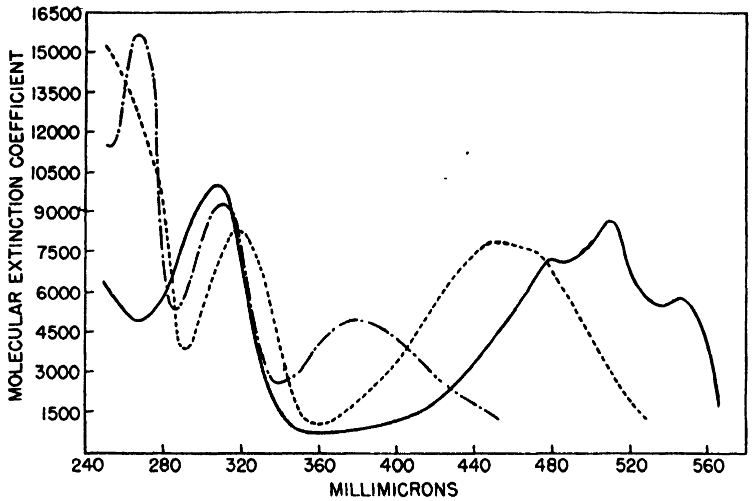
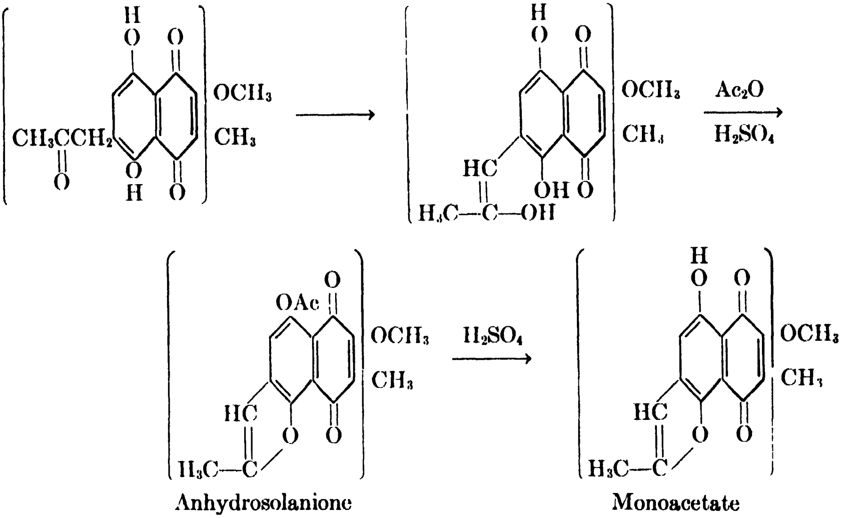


Fig. 7. Ultraviolet curves of: ——— Solanione; - - - - - Anhydrosolanione; —·—·— Anhydromonoacetate.

The monoacetate does not react with carbonyl reagents and gives none of the tests for a hydroxyl group. This suggested that the cleavage of water occurred between the carbonyl and a hydroxyl group. Further support for this reasoning came from the hydrolysis of the acetate with strong acid whereby there resulted an orange compound, $C_{15}H_{12}O_5$

(m.p. 246°C.) differing from solanione by the elements of water. The infrared spectrum of this substance (Fig. 4) showed the absence of the carbonyl absorption band originally present in solanione at about 5.83 μ. It is to be noticed that with both the monoacetate and the anhydro compound, the quinone carbonyl band is shifted to a slightly lower wavelength (5.96 μ). The formation of the monoacetate may be represented as follows:



Scheme II

and this type of ring closure is in agreement with that proposed by Arnstein and Cook (19) for the acetylation of javanicin.

This furan ring is extremely stable to acids, but is very sensitive to alkali, the original carbonyl and hydroxyl groups reappearing on treatment with the latter. Of the two hydroxyl groups present in solanione, the one undergoing this ring closure is the hydroxyl group responsible for the solubility of solanione in alkali (only a mono salt is formed) as shown by the failure of the anhydro compound to be extracted immediately from organic solvents by alkali.

If solanione is dissolved in 0.5 *N* NaOH and the resulting solution refluxed, the methoxyl group present in the molecule is cleaved. This was indicated by the formation of methyl alcohol during the hydrolysis, and the absence of any methoxyl group in the end product. A saponification of an ester grouping did not occur, since not only are all the oxygens accounted for, but also solanione is perfectly stable to acids under conditions which normally would hydrolyze any ester, if present. Furthermore, the resulting crimson product ($C_{14}H_{12}O_6$) forms an anhydrodiacetate, and the infrared spectrum of this new pigment shows, unlike solanione, an absorption band at 2.78 μ due to an unbonded hydroxyl group.

The ultraviolet (Fig. 8) and infrared (Fig. 6) absorption curves of this pigment are similar to those of solanione, indicating no fundamental change in structure during its formation. It differed from solanione in being soluble in $NaHCO_3$, and this increase in acidity placed the newly-formed hydroxyl group in the quinone ring.

There remained to be determined the location of the methyl group, and from chemical and physical data it is also placed in the quinone portion of the molecule.

Position of the Methyl Group

1. Chemical Evidence. As mentioned previously, solanione reacts with sodium hypioodite to yield iodoform and a quantitative investigation of this reaction revealed that 3 moles of iodoform are formed per mole of pigment reacting. One of these arises from the methyl ketone group and another could conceivably come from a methyl group located in the quinone ring. Support for this is the fact that phthiocol gave rise to one mole of iodoform when treated under the same conditions. Also, solanione gives a negative Craven test (20) indicating that the quinone ring was fully substituted.

2. Infrared Data. From a study of the infrared curves of naphthoquinone and 2-methylnaphthoquinone, the band attributed to the methyl group in the latter is thought to occur near 7.27 μ (14). A more detailed investigation of various methyl-substituted naphthoquinones suggests that, in addition to the band near 7.3 μ, there is another band near 7.8 μ which is also characteristic of the methyl group. Thus, a comparison of the infrared curves of naphthazarin and 2-methylnaphthazarin (Figs. 2,3) reveals that the latter contains 3 absorption bands at 7.32 μ, 7.72 μ, and 7.84 μ which are missing in the former. However, only the first and third bands are present in the other methyl quinones. In Table I are listed the bands which are thought to represent the absorption vibrations of the methyl group in these compounds. These bands are absent both in naphthoquinone (14) and in naphthazarin.

TABLE I

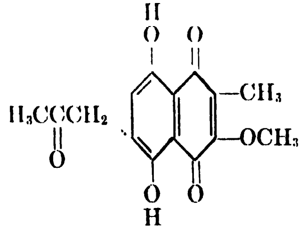
Absorption Bands Due to Methyl Group

Compound	Absorption band μ	
2-Methylnaphthoquinone	7.27	7.89 (estimated from graph (14))
Phthiocol (Fig. 2)	7.33	7.88
2-Methylnaphthazarin (Fig. 3)	7.32	7.84
Hydroxydreserone (Fig. 3)	7.32	7.89
2,6 (or 7)-Dimethyl-3,5,8-trihydroxynaphthoquinone (Fig. 5)	7.30	7.89
2-Methyl-6 (or 7) propyl-3,5,8-trihydroxynaphthoquinone (Fig. 5)	7.29	7.87
Demethylated solanione (Fig. 6)	7.37	7.89
Anhydromonoacetate (Fig. 4)	7.29	7.81
Anhydrosolanione (Fig. 4)	7.26	7.93

The one characteristic that all the synthetic naphthoquinones listed in Table I have in common, is the presence of a methyl group in the quinone ring. Since the various solanione derivatives (Table I) give rise to the two absorption bands near 7.3 μ and 7.8 μ, the data obtained serve as additional evidence in favor of the suggestion that the methyl group in solanione is located in the quinone ring.

3. Ultraviolet Data. Finally the close similarity of the ultraviolet curves of synthetic naphthoquinones containing both methyl and hydroxyl groups in the quinone ring with that of solanione or the demethylated compound lends added support for this contention (Fig. 8). This will be discussed later in more detail.

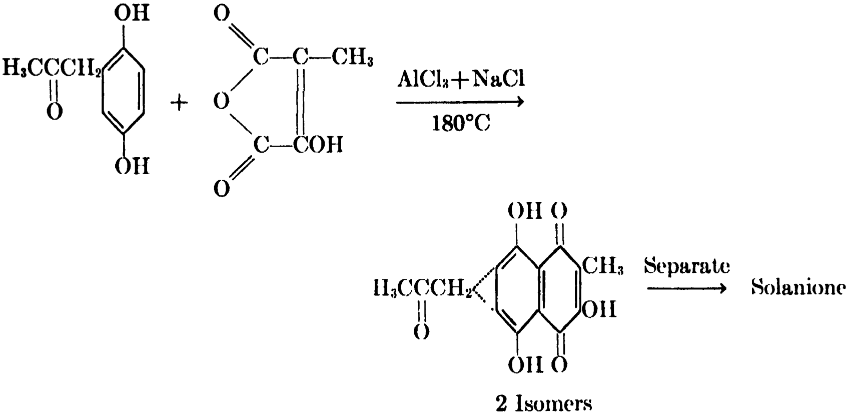
Thus, from the evidence presented, the probable structure of solanione can be represented as follows:



e. g., 2-methyl-3-methoxy-5,8-dihydroxy-6(or 7)-acetonyl-1,4-naphthoquinone.

ATTEMPTS AT SYNTHESES

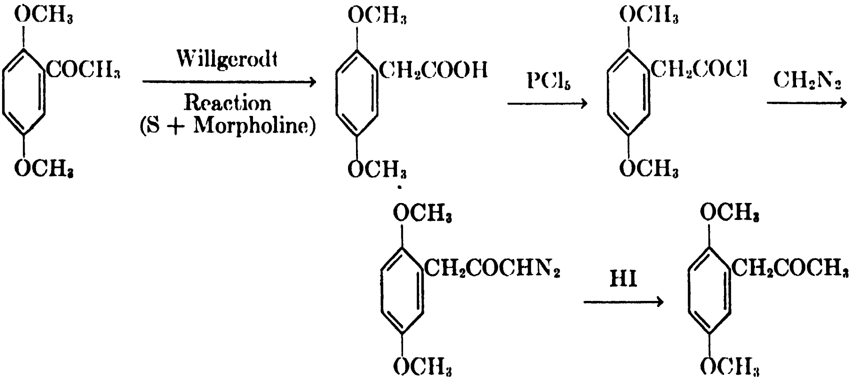
The usual procedure for the synthesis of complex naphthoquinones is the condensation of a substituted maleic anhydride derivative with the appropriate aromatic compound in the presence of an $AlCl_3$–NaCl melt. Thus, a proposed synthesis of solanione can be outlined as follows:



2,5-Dihydroxyphenylacetone   Hydroxycitraconic anhydride

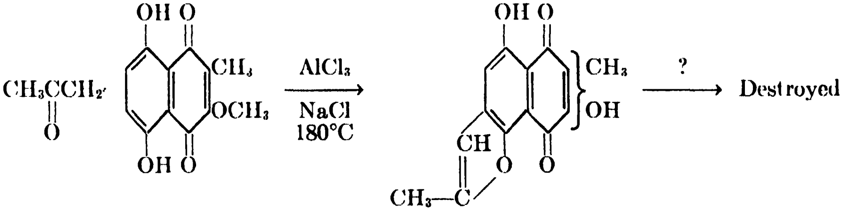
SCHEME III

Neither of these two compounds were known and it was decided to prepare the aromatic portion first since no information was available as to the stability of an acetonyl side group under such vigorous condensation conditions. 2,5-Dihydroxyphenylacetone (dimethyl ether) was therefore prepared as follows:



SCHEME IV

When trial condensation reactions were carried out, using this compound and maleic or citraconic anhydride, it was found that the expected product was formed in extremely poor yields. Further investigations seemed to indicate that the acetonyl side group was destroyed under these experimental conditions. As a matter of fact, when solanione was added to a NaCl–AlCl$_3$ melt, no original pigment was recovered, although a minute fraction (insufficient for analysis) was obtained, which, from its color and ultraviolet absorption curve, resembled the anhydro pigment. Since AlCl$_3$ is a strong dehydrating agent, such a ring closure is not unexpected; *e. g.*,

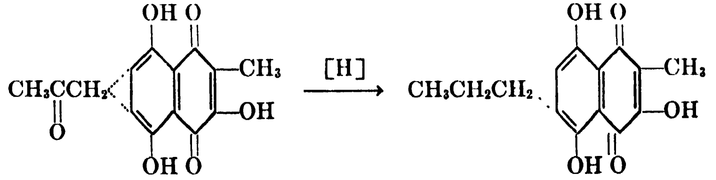


Solanione Anhydrocompound

Scheme V

Attempts were next made (using 2,5-dimethoxyphenylacetone and maleic anhydride) to vary the experimental conditions by employing other condensing agents (*e. g.*, $H_2C_2O_4$ and H_2SO_4), lower temperatures, or shielding the carbonyl group (*e. g.*, oxime), but all were of no avail.

From the above results, it did not seem feasible to synthesize solanione directly as proposed previously. If, however, the carbonyl group in the acetonyl side chain of demethylated solanione or solanione itself could be reduced, then the resulting compound (II),

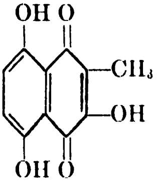


I II

Scheme VI

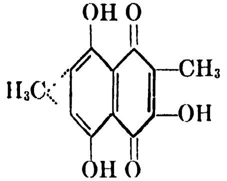
which now contains a more stable aliphatic side chain, might be obtained synthetically. This was found to be true, for compound (II) could be prepared, but attempts at reducing the saponified pigment (I) to (II) failed. The Wolf-Kishner reduction, or several modifications thereof, did not succeed because the pigment was destroyed by the alkali under the experimental conditions employed. The Clemmensen reduction gave only a gum, which was insoluble in alkali and organic solvents, while on catalytic hydrogenation one mole of hydrogen was absorbed (with the formation of the hydroquinone) and a second mole was taken up very slowly, presumably forming the hydroxy derivative.

Since the only difference between the synthetic compound (II) and the saponified pigment (I) is the presence of a carbonyl group in the side chain of the latter, it was decided to compare the curves of the ultraviolet and infrared spectra of (II) and several close analogues (III and IV (21)) with that of demethylated solanione (I).



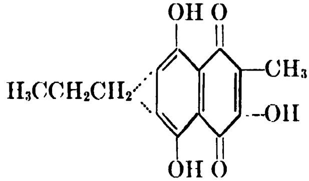
IV

Hydroxy-
dreserone



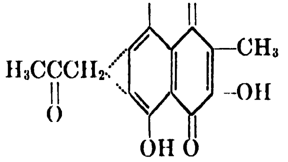
III

2,6(or 7)-Dimethyl-
3,5,8-trihydroxy-1,4-
naphthoquinone (2 isomers)



II

2-Methyl-3,5,8-trihydroxy-
6(or 7)-propyl-1,4-
npahthoquinone (2 isomers)



I

Demethylated solanione

In Fig. 8 are presented the curves of the ultraviolet spectra of (I), (II), and (IV). It should be mentioned that, in the preparation of (II) and (III), two isomers are obtained, but no attempt was made to separate them.

It can be seen that the closest similarity is between (I) and (II), and Table II showing the shift in wavelength of the maxima as one proceeds from (IV) to (I) is in agreement with this conclusion.

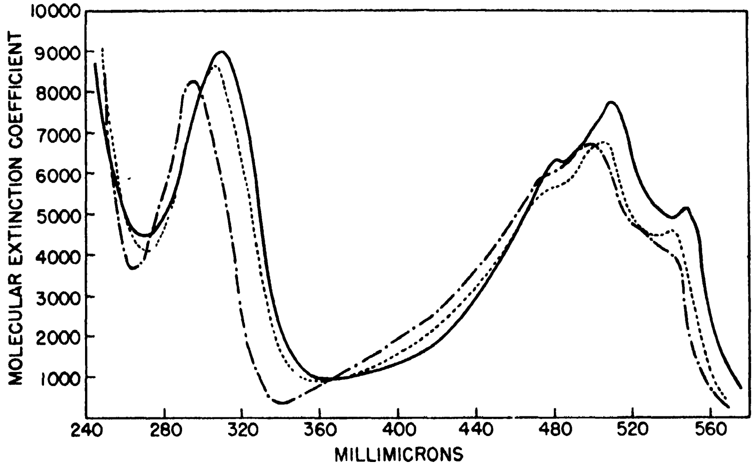


FIG. 8. Ultraviolet absorption curves of: ———— Demethoxylated solaniones; ------ 2-methyl-3,5,8-trihydroxy-6 (or 7)-propyl-1,4-naphthoquinone; —·—·— Hydroxydreserone.

TABLE II

Wavelength Shift of Related Compounds

Compound	λ maxima in ultraviolet region	λ maxima in visible region		
	mμ	mμ	mμ	mμ
IV	295	—	500	—
III	305	—	502	—
II	307	—	505	540
I (demethylated solanione)	310	482	510	548

Support for the proposed structure of solanione may be derived also from the comparison of the infrared spectra of compounds (II), (III), and (IV) with that of (I) or solanione (Figs. 3,5,6).

STUDIES ON THE MECHANISM OF PIGMENT FORMATION

To gain some insight into a possible mechanism of the genesis of solanione, it was of interest to investigate the formation of the pigment from sources other than glucose. At the same time, qualitative observations were made regarding the effect of various concentrations of iodoacetic acid, dimedon, and NaF on pigment production since these substances are known to interfere with the dissimilatory processes of yeasts and certain molds.

In these experiments, glucose, xylose, ethanol, and sodium acetate were used as substrates, the FsD pigment being obtained from the first 3 substances but not from sodium acetate. Growth and pigment production was unimpaired in the glucose solutions containing iodoacetic acid at concentrations up to 18.6 mg./l. Increasing concentrations of NaF, up to 8 m*M*, also had no effect on the growth and metabolism of FsD (glucose as substrate). A similar but not as marked effect had already been observed on yeast by Effront (21a). An even better growth and pigment production was observed, both on the media containing xylose and xylose plus dimedon (0.5%). However, in the case of glucose, those media to which dimedon (up to 0.2%) was added gave slower growth with less formation of pigment. In the flasks containing alcohol plus dimedon, crystals of the dimedon-acetaldehyde complex could be recognized easily. These were later separated and identified, thus indicating that the ethanol was being dehydrogenated by FsD to acetaldehyde. Moreover, of all substances tested, pigment production was first noticeable with alcohol (at least 48 hr. prior to any of the other carbon sources). Finally in the culture media containing ethyl alcohol plus dimedon, the appearance of pigment (as shown by the color) was delayed as compared with those containing ethanol alone.

EXPERIMENTAL

Cultures of *Fusarium solani* D_2 purple (*var. rosa*) obtained from the College of Agriculture, University of California, Berkeley 4, Calif., through the courtesy of Dr. W. C. Snyder, were used as a pigment source in these experiments. The stock cultures were maintained on the following medium:

20.00	g.	agar
83.50	g.	glucose
8.35	g.	sodium nitrate
8.35	g.	potassium phosphate (primary)
0.84	g.	magnesium sulfate (7 H_2O)
0.03	g.	ferrous sulfate
0.003	g.	zinc chloride
1000	ml.	distilled water

and periodically examined to check their purity. In this connection, it was observed that this culture of FsD gradually lost its ability to produce solanione, so that, after 9 months, no further quantities of the pigment were obtained. A new culture, received from the same source, again gave solanione under the same experimental conditions.

For the optimum-pH experiments, the molds were grown in test tubes. After two weeks, they were filtered, the filtrates acidified to pH 2, and read in an Evelyn colorimeter using a 520 mμ filter. The other preliminary experiments were run in 125 ml. Erlenmeyer flasks, using 50 ml. of medium.

For the large-scale experiments, the same medium as described above was employed except for the agar, which was omitted. Three liter Fernbach flasks were used for the growth of the mold, one liter of medium being placed in each and the solution sterilized at 120°C. under 15 lb. pressure for 20 min. Inoculation was made by means of a spore-mycelial suspension of cultures grown on the stock medium containing 2% agar. After one month's growth, the mycelia were separated from the solution by filtration, air-dried, and ground in an appropriate mill. The mycelia were then extracted in a Soxhlet for one week using petroleum ether (b.p. 30–60°C.), and the crude product which had precipitated out from the solvent during this interval filtered off. The best solvents for recrystallizations were found to be either acetone or alcohol (95%), from which solanione could be obtained in a pure state as a light red (acetone) to orange (alcohol) product, the latter assuming frequently a coppery luster, m.p. 208–210°C. (dec.) (depending on the rate of heating).

Analyses: Calculated for $C_{15}H_{14}O_6$: C, 62.1; H, 4.83; OCH_3, 10.7. MW: 290. Found: C, 62.4, 62.2; H, 5.11, 4.92; OCH_3, 10.1. MW: 284 (Rast).

Monopotassium Salt. To 50 mg. of solanione in a beaker was added an excess of alcoholic KOH (0.5 *N*). The orange pigment immediately turned blue-violet and the resulting mixture was filtered, washed copiously with absolute alcohol, and finally with absolute ether. The salt was dried *in vacuo* at 78°C. in a drying pump.

Analysis: Calculated for $C_{15}H_{13}O_5(OK)$: K, 11.9; found: K, 12.2.

Anhydromonoacetate. To 100 mg. of solanione in a beaker, there was added 2 ml. of acetic anhydride and 1 drop of conc. H_2SO_4. There was an immediate reaction as shown by the formation of a dark-red solution with the subsequent generation of heat. On standing for 1 min., the whole solidified to a dirty-yellow solid. Either the mixture can be poured into ice water from which, on standing, there is deposited a yellow solid, or else it can be recrystallized directly from boiling alcohol. The crude product so obtained was recrystallized twice from absolute alcohol from which it was obtained in light yellow needles, m.p. 252–253°C. (dec.), yield: 87 mg.

Analyses: Calculated for $C_{17}H_{14}O_6$: C, 65.0; H, 4.46; Acetyl, 13.7. Found: C, 65.1; H, 4.57; Acetyl, 13.1.

Anhydrotriacetate. This reductive acetylation was carried out essentially by the method described in the literature (23). Fifty mg. of the anhydromonacetate was added to a mixture of 1 g. of zinc dust, 100 mg. of fused sodium acetate, and 5 ml. of acetic anhydride and the whole heated gently until the yellow color could no longer be discerned (about 3–5 min.). Ten ml. of hot glacial acetic acid were then added and the mixture filtered at the boiling point and the residue washed further with more acetic acid. The excess acetic anhydride was destroyed by the addition of water, and on further dilution a light tan precipitate was obtained. This had a melting point of 259–

260°C. (dec.) and, on recrystallization from acetic acid-water, there resulted a colorless white product, m.p. 262–263°C. (dec.), yield: 48 mg.

Analysis: Calculated for $C_{15}H_{11}O_2(OCOCH_3)_3$: Acetyl, 32.3; found: Acetyl, 30.6.

2,4-Dinitrophenylhydrazone. This derivative was formed in the usual way using 50 mg. of solanione, 50 mg. of 2,4-dinitrophenylhydrazine, 10 ml. of alcohol and a few drops of conc. HCl. The crude product so obtained was recrystallized from chloroform-alcohol, m.p. 258–259°C. (dec.).

Analysis: Calculated for $C_{21}H_{18}O_9N_4$: C, 53.6; H, 3.83. Found: C, 53.5; H, 3.97.

Kuhn-Roth Oxidation. The procedure outlined by these investigators (10) was followed, and for every mole of solanione oxidized, 1.88 moles of acetic acid were found. *E. g.*, from 100 mg. of pigment, 39.0 mg. of acetic acid were obtained (by titration). Theoretical: 41.4 mg. acetic acid. That the only volatile acid formed was acetic acid was shown by the evaporation to dryness of the neutralized steam-distilled solution and the formation of the silver salt with alcoholic $AgNO_3$.

Analysis: Calculated for CH_3COOAg: Ag, 64.6; Found: Ag, 64.8.

Diboroacetate Complex. To a mixture of 250 mg. of solanione and 10 ml. of acetic anhydride in a test tube, there was added 400 mg. of pyroboroacetate (12), and the whole warmed at 50°C. for a short while. The solution turned a deep red and on cooling no product was obtained. The contents were placed in the refrigerator and 6 weeks later it was noticed that a precipitate had formed. This was filtered off, washed with acetic anhydride and absolute ether and analyzed by the procedure of Dimroth and Faust (12).

Analyses: 0.1513 g. of diboroacetate compound taken.

(a) Residue obtained: 77.1 mg.
calculated: 80.4 mg.
(b) Boric acid obtained: 30.5 mg. (by titration)
calculated: 34.4 mg.
(c) Acetic acid obtained: 62.2 mg. (by titration)
calculated: 66.6 mg.

Anhydrosolanione. To 50 mg. of the anhydromonoacetate was added 10 ml. of 50% H_2SO_4 and the mixture heated for 20 min. in a water bath. The orange residue, after filtration was recrystallized from benzene-petroleum ether from which the pure product was obtained, m.p. 246°C. (dec.).

Analysis: Calculated for $C_{15}H_{12}O_5$: C, 66.2; H, 4.42. Found: C, 66.6; H, 4.66.

Demethylated Solanione. Two hundred mg. of solanione was treated with 65 ml. of 0.5 *N* NaOH and the purple solution refluxed for 1.5 hr. The solution was cooled, acidified, and the deep-red precipitate filtered off. It was then dissolved in chloroform which was then extracted 8 times with 25 ml. portions of 5% $NaHCO_3$. The combined aqueous extracts were acidified with HCl, filtered, the residue redissolved in chloroform and dried over Na_2SO_4. On evaporation *in vacuo* of the chloroform, a bright crimson product, was obtained which, after one recrystallization from benzene-petroleum ether, gave the pure compound, m.p. 187–188°C.

Analyses: Calculated for $C_{14}H_{12}O_6$: C, 60.9; H, 4.35; OCH_3, 0.0. Found: C, 60.7; H, 4.53; OCH_3, 0.0.

A specimen was saponified in the manner just described and the alkaline solution steam distilled. A portion of the distillate gave a positive color test for methyl alcohol. According to Feigl (24), this color test is quite specific for methanol.

Anhydrodiacetate. This compound was formed from the demethylated pigment in a similar way as described for the anhydromonoacetate. Upon recrystallization from ethanol, its m.p. was 206–207°C. (dec.).

Analyses: Calculated for $C_{18}H_{14}O_7$: C, 63.2; H, 4.09; Acetyl, 25.2. Found: C, 63.2; H, 4.29; Acetyl, 23.9.

Iodoform Formation. The method followed for quantitative iodoform determination is that of Birkinshaw and Raistrick (25). Fifty mg. of solanione was dissolved in 125 ml. of 0.1 *N* NaOH, and to the purple solution there was added 100 ml. of 0.1 *N* iodine solution. The purple color immediately disappeared and, after standing in the dark for 7.5 hr., during which time there was precipitation of iodoform, the solution was acidified and the excess iodine removed with thiosulfate, using starch as the indicator. The solution was then extracted 4 times with 100 ml. portions of ether and the combined ether extracts washed with distilled water until a negative halogen test was obtained. To the ethereal solution was then added 40.0 ml. of 0.5 *N* alcoholic KOH and 50 ml. of alcohol and the ether distilled off. The resulting solution was then refluxed for 2 hr., acidified with 6 *N* HNO_3 and the iodide ions present determined by the Volhard procedure. At the same time a blank run was made.

Analysis: Calculated for 3 moles of iodoform: 204 mg. of iodoform. Found: 228 mg. of iodoform.

Another sample of solanione was treated in the manner just described but in this case the iodoform which had precipitated was filtered off, recrystallized from dioxane-water, and, upon mixing with an authentic sample of iodoform, gave no lowering of the melting point.

Fifty mg. of phthiocol was treated in an identical manner as solanione and here 118 mg. of iodoform was obtained. Theoretical value for one mole: 105 mg. of iodoform.

2,5-Dimethoxyphenylacetone. 2,5-Dimethoxyphenylacetic acid was prepared by the Kindler modification of the Willgerodt reaction from 2,5-dimethoxyacetophenone (26). To 25 g. (0.13 *M*) of 2,5-dimethoxyphenylacetic acid dissolved in 75 ml. anhydrous chloroform there was added 25.6 g. (0.13 *M*) of PCl_5. There was an immediate evolution of HCl and the solution turned brown. After standing overnight, the chloroform was evaporated off *in vacuo* and the residue dissolved in anhydrous benzene. This was then added slowly to a solution of diazomethane in ether at 0°C. (prepared from 75 g .of N-nitrosomethylurea). Nitrogen was evolved and the solution allowed to stand at room temperature overnight. There was a light yellow precipitate on the bottom of the flask the next day but it was not separated from the solution. Instead, the ethereal mixture was evaporated *in vacuo*, the outside temperature not exceeding 30°C., and the residue dissolved in 100 ml. of chloroform. Wolfrom and Brown (27) had shown that the action of HI on sugar diazoketones resulted in the formation of the corresponding methyl ketones. The same procedure was applied in this case, and therefore to the diazoketone (in chloroform) there was added 108 ml. of 47% HI. An immediate evolution of nitrogen occurred with the subsequent production of iodine and heat. The solution was washed with water, $Na_2S_2O_3$ solution, again with water, and finally dried over $CaCl_2$. The chloroform was evaporated off, and the resulting brown liquid distilled *in vacuo*. At 150–153°C./6 mm. a light yellow oil was obtained. Weight: 8.8 g. Yield: 35% of theory.

The oil gave a positive iodoform test and formed a $NaHSO_3$ addition product.

Oxime: m.p. 95.5–96.5°C.

Analysis: Calculated for $C_{11}H_{15}O_3N$: N, 6.69; found: N, 6.46.

Semicarbazone: m.p. 133–134°C.

Analysis: Calculated for $C_{12}H_{17}O_3N_3$: N, 16.72; found: N, 16.57.

The preparation of phthiocol, naphthazarin, methylnaphthazarin, and hydroxydreserone are described elsewhere (28).

2,6(or 7)-Dimethyl-3,5,8-trihydroxy-1,4-naphthoquinone. (Compound III). One g. of citraconic anhydride and 400 mg. of 2-methoxy-3,6-dihydroxy-toluene (21), were mixed thoroughly and added to a melt consisting of 10 g. $AlCl_3$ and 2 g. NaCl (temperature of melt: 180°C.). The melt now had a red color, and after keeping the temperature at 200°C. for 2 min., the temperature of the melt was raised to 220°C. and then allowed to cool. The red solid was treated with water and, to the resulting purple solution, there was added an equal volume of conc. HCl. The whole was boiled for a few minutes, cooled, and filtered. Both the filtrate and the residue were extracted thoroughly with chloroform, the latter by means of a Soxhlet. The combined chloroform layers were treated with 100 ml. portions of 2 *N* NaOH until colorless. The alkaline solution (red) was acidified and the light orange amorphous precipitate filtered. Yield of crude product was 155 mg. A portion was sublimed *in vacuo* (at 140°C. and 20 mm. pressure) yielding a bright crimson sublimate.

Analysis: Calculated for $C_{12}H_{10}O_5$: C, 61.5; H, 4.27. Found: C, 61.7, H, 4.30.

2-Methyl-3,5,8-trihydroxy-6 (or 7)-propyl-1,4-naphthoquinone (Compound II). This compound was prepared in the same way as that described for compound III except that propyl maleic anhydride was used instead of citraconic anhydride. The crude product was sublimed, and a bright crimson sublimate obtained.

Analysis: Calculated for $C_{14}H_{14}O_5$: C, 64.1; H, 5.34; found: C, 64.4; H, 5.18.

Data on Infrared Spectra

An experimental model of the Baird Double-beam infrared recording spectrophotometer was used.[2] With the exception of solanione, the anhydro compound and the anhydromonoacetate, all the naphthoquinones were dissolved in CCl_4, the concentration being approximately 5 mg. of compound/2 ml. of solvent. Warming was required in all cases to effect solution. In the case of the 3 substances which did not completely dissolve in the CCl_4, the infrared curves were run on the material (5 mg.) suspended in the solvent (*ca.* 2 ml.). A 1 mm. liquid cell with NaCl windows was employed.

The data in the region of solvent absorption (6.2–6.7 μ, 7.9–8.3 μ, and 9.7–10.3 μ) must be interpreted with great care because of lowered energy and, hence, instrument sensitivity in these regions. These curves show solvent absorption as deflections to higher transmission because the comparison cell is slightly thicker than the sample cell. The wavelength calibration of this instrument is within 0.04 μ. All curves were run under similar operating conditions.

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[2] We wish to thank Mr. Carl C. Clark and Dr. James D. Hardy of the Department of Physiology, Cornell University Medical School, for their courtesy and the facilities placed at our disposal.

Data on Ultraviolet Spectra

All curves were obtained by using a Beckman spectrophotometer, and with the exception of the case of the anhydromonoacetate and the anhydrosolanione, the solvent was chloroform. With the other compounds, 95% alcohol was used.

COMMENTS

The exact position of the acetonyl group in the FsD pigment is not known. Although the melting points of solanione and its various derivatives are in the same vicinity as those of javanicin (19,22) (being consistently higher in the case of solanione), there are certain differences between them which indicate that these compounds may not be identical with each other. Thus, according to Arnstein and Cook (19), javanicin does not contain any easily hydrolyzable group while with solanione the methoxyl group can be readily split off with alkali. While the absorption curve of javanicin contains only one maximum (at 510 mμ) in the visible region (using chloroform as the solvent), solanione contains three *distinct* maxima (at 480 mμ, 509 mμ and 546 mμ) in this region. That these other two maxima (at 480 mμ and 546 mμ) are real, is shown by the fact that, with the saponifiied pigment, three maxima are also obtained, being located in practically the same place as those of solanione (*e. g.*, 482 mμ, 510 mμ and 548 mμ). Furthermore, the synthetic compound (II) (see p. 305) also shows a maximum in addition to that at 505 mμ (*e.g.*, at 540 mμ). Moreover, the absorption curve of the javanicin anhydro compound contains a maximum at 264 mμ which is consistently absent in the case of anhydrosolanione.

There are some indications that one of the intermediates involved in the formation of solanione and related pigments may be acetaldehyde. Thus, pigment formation appears earlier when alcohol is used as the substrate than when either glucose or xylose is employed as the carbon source. The formation of the dimedon derivative from alcohol shows that FsD is capable of dehydrogenating the alcohol to acetaldehyde. The mold would require a longer period of time to reach the acetaldehyde stage from xylose or glucose than from alcohol. Furthermore, in the presence of alcohol and dimedon, pigment production was not as rapid and extensive as with alcohol alone, indicating that less acetaldehyde was available for pigment synthesis. Moreover, grown on glucose plus dimedon, FsD shows a slower rate of pigment formation than on glucose alone.

That acetaldehyde could act as the key intermediate for the synthesis of a wide variety of compounds was visualized as early as 1919 (29) and demonstrated recently, for example, in the methyl-*p*-methoxycinnamate (30) or ergosterol (31) formation by other molds. Furthermore, the fact that FsD is an alcoholic fermenter, and that it can tolerate concentrations of fluoride which normally inhibit yeast fermentation (32), suggests that the acetaldehyde is formed by a phase sequence other than that involving phosphorylated intermediates.

SUMMARY

The conditions for growth and isolation of solanione, a pigment from *Fusarium solani* D_2 purple are described.

Through certain chemical reactions and degradation studies, as well as infrared and ultraviolet absorption spectra, solanione is thought to be 2-methyl-3-methoxy-6 (or 7)-acetonyl-5,8-dihydroxy-1,4-naphthoquinone.

A possible mechanism for the formation of solanione and related pigments from acetaldehyde is discussed.

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On the Mechanism of Enzyme Action. XXXVIII. Effect of Different Naphthoquinones on the Fat Formation in *Fusarium lini* Bolley

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INTRODUCTION

Previous work from this laboratory (1,2) revealed that when solanione, a substituted naphthoquinone obtained from *Fusarium solani* D_2 purple (FsD) (3), is added to culture media of a growing non-pigment producer, *Fusarium lini* Bolley (FlB), the carbohydrate conversion factor was greatly lowered and the qualitative composition of the fat obtained was also changed. To determine whether such effects were attributable to solanione only, a series of structurally related naphthoquinones were prepared, and their influence on fat formation in FlB at different concentrations studied. At the same time, we attempted to ascertain a correlation, if any, between structure and action of these compounds.

EXPERIMENTAL

The same FlB culture and Raulin-Thom medium as described previously (1) were used, the concentration of glucose in the latter being 2.5%. With the exception of 1,4-dihydroxynaphthalene, the pigments were dissolved in acetone and added to the medium before sterilization, the solvent being allowed to evaporate prior to addition of the nutrient solution. The dihydroxynaphthalene was dissolved in alcohol and added with aseptic technique to the sterilized medium. The mold was grown in 3-liter Fernbach flasks, 1 l. of solution being added to each. All series were set up in quadruplicate and the flasks inoculated with 5 ml. of a spore-mycelial suspension. After 3 weeks, the media were filtered and the contents and mycelia of each series combined. The solutions were analyzed for sugar (4), and fat determinations were carried out as previously described (5). The amount of nitrogen in the dried mycelia was established by the standard microKjeldahl method. Oxidation-reduction potentials of solanione and its various derivatives were measured in the customary way (6).

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The naphthoquinones used in this study were the following: (a) 1,4-naphthoquinone (commercial), recrystallized twice from ethanol, m.p. 125–126°C. (b) 2-methyl-1,4-naphthoquinone (commercial), recrystallized from ethanol, m.p. 104–105°C. (c) Phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) was prepared from 2-methyl-1,4-naphthoquinone (7), m.p. 172–173°C. (d) 1,4-dihydroxynaphthalene was prepared from pure α-naphthoquinone by reduction with $SnCl_2$ and HCl, filtering the hot colorless solution from which, on cooling, there is deposited the desired compound in white needles, m.p. 175–176°C. (e) Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone was synthesized by the procedure of Macbeth *et al.* (8), using maleic anhydride and hydroquinone. Final purification was carried out by sublimation *in vacuo.* (f) Methylnaphthazarin (2-methyl-5,8-dihydroxy-1,4-naphthoquinone) was prepared in the same manner as naphthazarin, except that the compounds to be combined were citraconic anhydride and hydroquinone. The crude product so obtained was purified by sublimation *in vacuo.* (g) Hydroxydreserone (2-methyl-3,5,8-trihydroxy-1,4-naphthoquinone) was prepared by the method of Winzor (9). This compound was also purified by sublimation *in vacuo.*

The preparation of solanione, the anhydromonoacetate, anhydrosolanione, and the demethylated solanione were described elsewhere (3).

DISCUSSION

The results are presented in Tables I and II.

Certain generalizations can be made regarding the action of all the naphthoquinones. Thus, in general, the pigments at their respective concentrations decreased the mycelial weights as compared to the controls from 13% as shown by the demethylated solanione to 100% (or no growth at all) as shown by naphthazarin at the 4 mg. level.

As observed in previous experiments (1), the higher the concentration of pigment within a given series, the more glucose remains unutilized. It will be noticed from the data recorded in Tables I and II that, at the 1 mg. level of pigment, usually no glucose is found at the end of the 3 week growth period. However, in all cases, the average mat weight has decreased indicating that, although the sugar is consumed (as with the controls), synthetic activity has diminished. This is further borne out by the lower values obtained for the fat coefficients as compared with the control, indicating that, in the presence of these compounds, the conversion of carbohydrate to fat is affected. In previous work, it was noticed that solanione is instrumental, on the one hand, in increasing the rate of certain dehydrogenating processes in FlB as shown by the formation of a fat containing more linoleic acid than the control (2), and, on the other hand, inhibiting others as indicated by the de-

TABLE I

Effects of Synthetic Naphthoquinones on Fat Formation in FlB

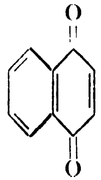
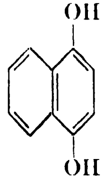
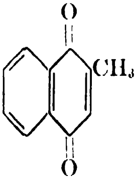
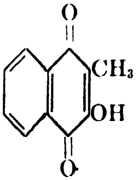
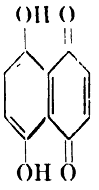
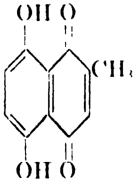
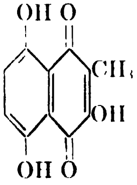
Compound	Quantity pigment	Average mat weight	Difference of mycelial wts from control	Unutilized glucose	Nitrogen	Total lipide	Fat coef[a]	Oxidation-reduction potential[b]
	mg./l.	*g.*	*per cent*	*g./l.*	*per cent*	*per cent*		*millivolts*
Control	0	4.83	0	0.0	7.21	7.76	1.50	
 1,4-Naphthoquinone	1	2.60	− 46	0.0		6.90	0.72	488
	2	2.59	− 46	0.8		7.02	0.75	
	4	1.78	− 63	1.6	6.24	6.85	0.52	
 Hydronaphthoquinone	1	2.80	− 42	0.0		6.52	0.72	
	2	2.46	− 49	0.6		6.57	0.67	
	4	1.78	− 63	1.3	6.06	6.50	0.49	
 2-Methyl-1,4-naphthoquinone	1	1.84	− 62	0.8		6.17	0.47	472
	2	1.63	− 66	1.3		6.32	0.43	
	4	1.39	− 71	2.6	6.21	5.92	0.37	
 Phthiocol	1	1.78	− 63	1.8		8.04	0.62	306
	2	1.28	− 73	2.7		8.15	0.47	
	4	0.90	− 81	6.7	7.17	8.15	0.40	

TABLE 1—*Continued*

Compound	Quantity pigment	Average mat weight	Difference of mycelial wts. from control	Unutilized glucose	Nitrogen	Total lipide	Fat coef.[a]	Oxidation-reduction potential[b]
	mg./l	*g*	*per cent*	*g/l.*	*per cent*	*per cent*		*millivolts*
 Naphthazarin	1	2.74	− 43	1.8		5.75	0.68	376
	2	0.90	− 81	8.8		4.70	0.26	
	4	0.0	−100	24.0	0.0	0.0	0.00	
 2-Methylnaphthazarin	1	3.65	− 21	0.4		5.02	0.74	323
	2	3.21	− 33	0.7		5.21	0.70	
	4	3.24	− 33	1.4	6.64	3.70	0.51	
 Hydroxydroserone	1	3.23	− 33	0.0		6.58	0.85	210
	2	3.21	− 33	0.4		5.26	0.69	
	4	2.38	− 50	1.0	7.11	5.24	0.52	

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[a] The fat coefficient is defined as g. of total lipide produced/100 g. of carbohydrate consumed.

[b] These values are taken from the paper of Wallenfels and Mohle (6).

crease in the rate of dehydrogenation of isopropanol by FlB (1). It may be, however, that other types of enzyme systems in FlB are also affected by these substances. Thus, it has been shown that naphthoquinones are capable, for example, of influencing respiratory processes in bacteria (10,11) and plants (12), of inhibiting certain enzymes such as yeast carboxylase (13), succinic acid oxidase. and dehydrogenase

TABLE II

Effects of Solanione and Its Derivatives on Fat Formation in FlB

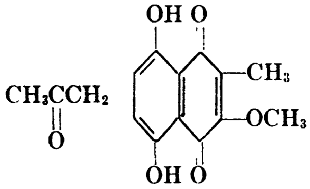
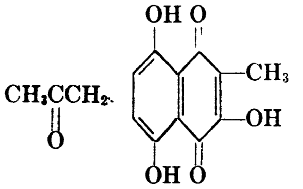
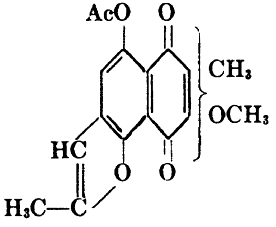
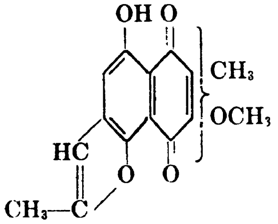
Compound	Quantity pigment	Average mat weight	Difference of mycelial wts. from control	Unutilized glucose	Nitrogen	Total lipide	Fat coef.	Oxidation-reduction potential
	mg l.	*g.*	*per cent*	*g l.*	*per cent*	*per cent*		*millivolts*
Control	0	4.83	0	0.0	7.21	7.76	1.50	
 Solanione	1	3.07	−37	0.0		6.29	0.77	291
	2	2.23	−53	0.0		6.18	0.55	
	4	1.66	−66	1.1	6.56	4.57	0.32	
 Demethylated solanione	1	4.13	−14	0.0		6.29	1.03	289
	2	4.13	−14	0.0		6.28	1.03	
	4	4.20	−13	0.5	7.30	6.14	1.05	

TABLE II—*Continued*

Compound	Quantity pigment	Average mat weight	Difference of mycelial wts. from control	Unutilized glucose	Nitrogen	Total lipide	Fat coef.	Oxidation-reduction potential
	mg. l	*g*	*per cent*	*g., l*	*per cent*	*per cent*		*millivolts*
 **Anhydromono acetate**	1	2.25	−53	0.0		5.86	0.53	370
	2	1.76	−63	0.6		5.88	0.42	
	4	1.60	−67	1.1	6.30	6.12	0.41	
 **Anhydrosolanione**	1	1.95	−60	0.0		6.20	0.48	295
	2	1.74	−64	0.5		6.15	0.44	
	4	1.75	−64	1.0	6.12	5.32	0.39	

(14), and of interfering with carbohydrate metabolism in certain parasites (15, 16).

It will be observed from the data recorded in Table I that, in the case of naphthoquinone and hydronaphthoquinone, the average mycelial weights, the nitrogen, the amount of fat formed, the unutilized glucose, and the fat coefficients are approximately the same for all 3 concentrations of the pigments. This suggests that the mold is either capable of converting one form completely to the other, in which it may function, or else the similarity in action is entirely fortuitous. This latter consideration is improbable as the two compounds are so different with respect to their chemical activities. The first consideration, on the other hand, is supported by the established presence of riboflavin in FlB (35 γ/g. mycelium, grown in the dark) which may act as a member of the hydrogen-transport system participating in these transformations. Also, with 2-methylnaphthoquinone, which is certainly closer in structure to naphthoquinone than hydronaphthoquinone, there are great differences in fat coefficient, average mycelial weights, and the amount of fat produced, from that of naphthoquinone. It appears more probable that the enzymes present in the mold dehydrogenated the hydronaphthoquinone to naphthoquinone, for the general results are in agreement with that obtained with the other quinones employed.

Since redox potentials generally give an indication of the oxidizing level of the respective quinones, it was of interest to search for any correlation between these potentials of the various naphthoquinones and their action *in vivo*. It can be seen from the data in Tables I and II, however, that no such relationship exists. Thus, solanione and its saponified product, demethylated solanione, have approximately the same redox potential, but their effects on FlB are greatly different from each other. For example, at the 4 mg. level, the fat coefficient of the latter is more than 3 times higher than the former.

It can be seen from the data recorded in Table I that the introduction of a methyl and a methyl and hydroxyl group into the quinone ring causes a greater decrease in both the mycelial weights and the fat coefficient as compared with naphthoquinone. For example, in the presence of 1 mg. of naphthoquinone, there is a decrease of 42% in mycelial weights as compared with the controls, while with phthiocol, at the same concentration, a decrease of 63% is obtained. With naphthoquinone, the fat coefficient is 0.72, while with phthiocol it is 0.62.

However, in the case of naphthazarin, the effect is more pronounced.

Here, at the 4 mg. level, no growth at all is obtained. This inhibitory action can be lessened by additional substitution in the quinone ring as illustrated by methylnaphthazarin and hydroxydreserone. Thus, in the presence of 2 mg. of naphthazarin, there is a decrease of 81% in mat weight while with methylnaphthazarin a decrease of only 33% is obtained. The fat coefficient for naphthazarin is 0.26, while for methylnaphthazarin it is 0.70.

With solanione and its derivatives, it was found (Table II) that if the methoxyl group is removed, the resulting compound, demethylated solanione, differs greatly in its action *in vivo* from that of its parent compound. Thus, for example, at the 2 mg. level, the average decrease in mat weight in the presence of solanione is 53% while with the demethylated compound, only a decrease of 14% was found (as compared to the controls). But of even more importance is the comparison of the fat coefficeints obtained using these two pigments. With the former (solanione), a fat coefficient of 0.55 is found, while with the latter it is 1.03. The anhydromonoacetate or the anhydrosolanione have a higher inhibitory effect than solanione.

Summary

1. Various naphthoquinones, when added to a growing non-pigment producer, FlB, cause a decrease in the fat coefficient, showing that they affect the enzyme systems involved in the conversion of carbohydrate to fat.

2. When both naphthoquinone and hydronaphthoquinone are used (in separate experiments), apparently the enzymes present in the mold first convert one to the other, exerting in this form its interaction during the growth of the mold. This may be part of the mechanism by which these pigments interfere in the action of the various enzyme systems concerned.

3. In all cases, there is a decrease in mycelial weights in the presence of these pigments in the culture media, the greatest effect being with 4 mg. of naphthazarin which resulted in complete inhibition.

4. An attempt has been made to correlate structure and function in the various naphthoquinones.

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Selenate Inhibition Studies. III. The Reversal of Selenate Inhibition in *E. coli*[1]

In a previous communication it was shown that methionine, but not cysteine or glutathione, was capable of partially reversing the toxic effect of selenate upon yeast growth (1). On the other hand, evidence presented below demonstrates that, for *E. coli*, cysteine and, to some extent, glutathione, but not methionine is utilized in nullifying the selenate effect.

H_2SeO_4	Cysteine	Glutathione	Methionine	Optical density
mg.	*mg.*	*mg.*	*mg.*	(2-log G)
1	0	0	0	0.00
1	0.5	0	0	0.83
1	1.0	0	0	0.86
1	2.0	0	0	0.89
1	0	1	0	0.50
1	0	2	0	0.53
1	0	0	1	0.00
1	0	0	2	0.00
1	0	0	4	0.00
3	0	0	0	0.00
3	0.5	0	0	0.58
3	1	0	0	0.80
3	2	0	0	0.80
3	0	1	0	0.08
3	0	2	0	0.15
3	0	0	1	0.00
3	0	0	2	0.00
3	0	0	4	0.00

The above substances are indicated as supplements added per tube. The "SG" medium of Harris and Kohn (2) was used, with a volume of 10 ml. of medium per tube, instead of 8. The optical densities represent

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[1] This work was supported by a grant from Swift and Co., Inc.
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growth at the end of 52 hr. at 37°C. Comparison with control tubes containing no selenate could not be made, inasmuch as growth in the selenate tubes was accompanied by a red coloration.

In a separate investigation, it has been found that 2-chloro-4-aminobenzoic acid, which is considered a specific inhibitor for methionine synthesis in *E. coli* (3), had no action against *Sacc. cerevisiae* in a medium containing sulfate as the sole sulfur source (4). In addition to this, it has been reported that cysteine but not methionine can serve as a satisfactory sulfur source for *E. coli* (5). We have found the reverse to be true for *Sacc. cerevisiae.*[2] It would appear from these observations that the synthesis of methionine from sulfate is accomplished by different routes in these two organisms.

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Acid-soluble Phosphorus Content of Embryos Infected with Influenza "A" Virus

Influenza virus produces important metabolic changes in fertile chicken eggs. Increases have been found in the volume of the allantoic fluid and in its pH, in the weight of the corioallantoic membrane, in the rate of anaerobic glycolysis, and in the oxygen uptake during the first hours of infection (1,2). The most notable change, however, is the arrest of their development (1,3), and it was considered that the metabolism of phosphorus might be related to this change On the other hand it was found that the total quantity of creatine and creatinine increases in the allantoic fluid after infection with influenza virus, the substance being presumably derived from muscle phosphocreatine (4).

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[2] Unpublished data.

MATERIAL AND METHODS

A local strain of influenza A virus was used (5). This virus was adapted to chicken embryo and a transfer made 3 days before the experiment. A mixture of virus with penicillin that contained 10^7 MID and 500 U. of penicillin in 0.1 cc. was introduced. The controls were inoculated with the same quantity of normal allantoic fluid and an equal quantity of penicillin.

The eggs were from Leghorn chickens, and had been incubated 12 days at 38°C. before the inoculation. Eggs weighing 58–60 g. were chosen since it was found that some of the variations were not clearly discernible using smaller eggs.

The eggs were inoculated in the allantoic sac and incubated at 37°C. The tests were carried out at 2, 4, 7, 24, and 48 hr. after inoculation. The embryos were lifted out with forceps and placed immediately in a mixture of petroleum ether and dry ice. They were stored in a CO_2 cabinet until required. The "true" inorganic phosphorus was determined by the Lowry and Lopez (6) method, whereas Fiske and SubbaRow's (7) method was used to measure the total inorganic phsophorus, that hydrolyzable in 7 min. at 100 °C. and the total acid-soluble P.

RESULTS AND DISCUSSION

Two hr. after inoculation, the values for the various P fractions in the embryos infected with the influenza virus were identical with those for

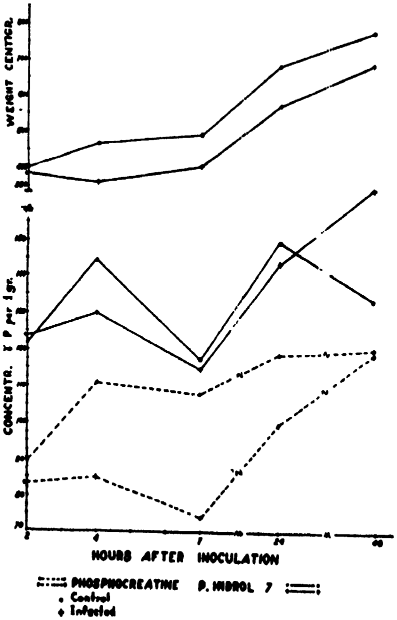

FIG. 1. Weight of chick embryo in cg. Concentration of phosphocreatine and P hydrolyzable in 7 min. in γ/g. of tissue.

TABLE I

Concentration of the Phosphorus Acid-Soluble Fractions
(in γ/g. of tissue)

		Hr. after inoculation																			
		2				4				7				24				48			
		No. det.	Av.	σ^c	$\frac{\Delta^a}{\text{S.E.}}$	No. det.	Av.	σ	$\frac{\Delta^a}{\text{S.E.}}$	No. det.	Av.	σ	$\frac{\Delta^a}{\text{E.S.}}$	No. det.	Av.	σ	$\frac{\Delta^a}{\text{E.S.}}$	No. det.	Av.	σ	$\frac{\Delta^a}{\text{E.S.}}$
Weight (cg.)	N[b]	18	400	50	<1	29	461	66	6.4	23	488	63	4.1	8	688	65	3.69	8	769	130	2.10
	I[b]	18	388	67		29	358	54		23	403	76		8	566	66		8	685	99	
"True" inorganic P	N	18	163	43	<1	27	157	41	1.0	22	171	40	2.0	8	265	45	2.3	8	271	64	<1
	I	18	157	29		28	145	43		23	146	43		7	205	53		8	265	38	
Phosphocreatine	N	18	88	32	<1	27	111	47	2.2	22	108	36	3.08	8	119	46	<1	8	120	66	<1
	I	18	83	43		28	85	38		23	71	29		7	101	44		8	119	33	
P hydrol. in 7 min.	N	18	122	18	<1	29	146	77	1	22	116	24	<1	8	150	20	<1	8	133	23	1.54
	I	18	124	32		29	130	30		23	114	17		7	144	16		8	164	51	
P acid-sol. Total	N	12	597	81	1.42	22	545	79	2.3	15	541	56	1.8	8	761	159	<1	8	770	113	<1
	N	12	555	61		20	498	50		15	503	55		8	702	63		8	763	156	

[a] Δ = Difference between the means; S.E. = Standard error of the difference.
[b] N, Control; I, Infected with virus.
[c] Standard deviation.

the controls. At 4 hr., there was a clear diminution in the phosphocreatine in the infected embryos, which was accentuated at 7 hr. and became normal again at 24 hr. The phosphorus and the total acid-soluble phosphorus diminish, although this decrease does not show the same intensity and persistency as the phosphocreatine. At the same time, the embryo shows a decline in weight (Fig. 1, Table I).

The decrease in the phosphocreatine with normal values of the P hydrolyzable in 7 min. may be similar to that detected in the period of anaerobic activity of the muscular contraction and also if the muscle is poisoned with monoiodoacetic acid. In both cases the ADP is converted into ATP at expense of the phosphocreatine without temporary resynthesis of the latter.

Experiments are in progress to determine the truth of these hypotheses.

Conclusions

Infection of fertile eggs with a large dose of influenza A virus produces in the embryo an early drop in weight together with a simultaneous reduction in the concentration of phosphocreatine.

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[1] With the technical assistance of M. Cosmelli.

Calcium and Apyrase System of Muscle

Calcium has been noted as an activator of ATPase associated with myosin (1), and its effect has been suggested as diagnostic of ATPase systems in general (2). On the other hand, hydrolysis of ATP by whole frog muscle homogenates is not activated by calcium and may even be depressed (3). This behavior has been noted for other tissue homogenates as well (4). Preliminary experiments suggested that calcium activation was associated with solution of the enzymes in the usual strong KCl extractives (3).

Thigh muscles of grass frogs were homogenized in the cold (0 to about 6°C.) with about 10 times their weight of glass-distilled water using all glass homogenizers. The pH of such homogenates ranged from 6.2 to 6.9 as measured with the glass electrode. Apyrase activities were measured, by methods previously described, at 19°C., using 10 min. incubation times. Substrate (Na salt of ATP, Rohm and Haas) was added in amounts such that less than one half of the 7 min. phosphorus was hydrolyzed during the test period.

Fractionations were carried out in the cold by centrifuging as noted. Precipitates were resuspended with the aid of a glass homogenizer, to final volumes proportional to the amounts of original suspension being used.

Table I records two typical runs on the frog muscle homogenates. The following points may be noted: 1. Somewhat less than half of the total nitrogen is water-soluble, while only around a tenth of the apyrase activity is extracted. Water-soluble enzyme is inhibited by Ca^{++} and less so by Mg^{++}. 2. Re-extraction with water removes virtually no more nitrogen or enzyme. 3. Water-insoluble enzyme shows little activation or inhibition with Ca^{++} or Mg^{++}. 4. Treatment of water-insoluble enzyme with 0.5 *M* KCl depresses the activity in the absence of calcium, but calcium and magnesium both activate back to at least the level of the water suspension. 5. Enzyme soluble in 0.5 *M* KCl is strongly activated by calcium, less so by magnesium. The insoluble residue shows enzyme activity but relatively small effects of the ions. 6. The KCl-soluble fraction shows the highest activity per unit of nitrogen in the presence of calcium. 7. The sum of the activities of KCl-soluble and insoluble fractions is distinctly less than the whole KCl suspension activity in the absence of calcium. 8. A strongly Mg-activated fraction is not apparent (5) although the obsrvations do not preclude its existence.

TABLE I

Apyrase Activities of Various Fractions of Frog Muscle Homogenates as Per Cent Total Original Homogenate and as $\gamma P/mg.\ N/min.$ at 19°C

Reaction volumes of 2 ml. Each tube contained, in final concentration, ATP (170 γ/g 7 min. P), veronal buffer 0.02 *m*, KCl 0.025 *m* (added from stock or as KCl extract), enzyme preparation and $CaCl_2$ or $MgCl_2$ as noted, 5×10^{-3} *M*. Reaction times: 10 min. Negligible activity with glycerophosphate as substrate. Values marked * were corrected for about 20% loss of N due to incomplete washings. Ca^{++} and Mg^{++} in higher concentrations (5×10^{-2} *M*) either activated less or inhibited more, the qualitative effects being the same as those for the lower concentrations. Supernatant and residue refer, respectively, to fractions collected following centrifugation for 10 min. at 27,000 *g*. Each extraction 30–60 min.

	N	Apyrase activity				$\frac{+Ca}{-Ca} \times 100$	$\frac{+Mg}{-Mg} \times 100$
		−Ca		+Ca		.005 *M*	.005 *M*
			$\gamma P/mg.\ N/min$		$\gamma P/mg\ N/min.$		
A. Muscle homogenate in H_2O	*per cent total*	*per cent total*		*per cent total*			
1.	100	100	18	100	16	86	90
2.	100	100	25	100	21	87	88
B. Supernatant of A							
1.	51	13	4	4	1	25	74
2.	34	11	8	8	5	62	100
C. Residue of A in H_2O							
1.	53	57	19	68	21	110	108
2.	54	52	27	67	28	104	104
D. Supernatant of C							
1.	9*	—	—	—	—		
2.	7	—	—	—	—		
E. Residue of C in H_2O							
1.	44*	54*	25	73*	27	108	107
2.	48	45	24	49	21	88	100
F. Residue of A in 0.5 *M* KCl							
1.	51*	43*	15	79*	25	167	129
2.	58	44	20	66	24	120	140

TABLE I—(*Continued*)

	N	Apyrase activity				$\frac{+Ca}{-Ca}\times 100$	$\frac{+Mg}{-Mg}\times 100$
		−Ca		+Ca			
			γP/mg. N/min.		γP/mg. N/min.	.005 *M*	.005 *M*
	per cent total	*per cent total*		*per cent total*			
G. Supernatant of F in 0.5 *M* KCl							
1.	27*	10*	6	59*	37	620	130
2.	22	2	6	18	41	680	300
H. Residue of F in 0.5 *M* KCl							
1.	24*	8*	6	12*	8	130	75
2.	32	12	19	15	25	130	110

From these findings it can be concluded that pronounced calcium activation of frog muscle apyrase follows the depression of activity occasioned by extraction with strong KCl. Further work will be necessary to decide whether this is a function of calcium bound to the insoluble portion, or whether the activity of the enzymes in the original preparation is due to structural relations with other organic components.

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Erratum

In Volume 20, Number 1, the January 1949 issue, page 168, line 6, "breakdown to triacetic acid lactone" should read "breakdown of triacetic acid lactone."

Spontaneous and Nitrogen Mustard-Induced Nutritional Deficiencies in *Saccharomyces cerevisiae* [1]

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INTRODUCTION

Saccharomyces cerevisiae is potentially better material for the study of biochemical genetics than organisms hitherto studied [see reviews by Beadle (1), by Bonner (2), and Lindegren (3)], since its enzymatic, constitution has been more thoroughly investigated, since it can be handled by convenient bacteriological techniques (for growth-rate measurements, plating procedures, *etc.*) and since, like many other *Ascomycetes*, it has a life cycle amenable to conventional genetic analysis (3,4).

The work reported here is concerned with the use of nitrogen mustard for the production of mutant strains of yeast with nutritional deficiencies of the types previously found in *Neurospora* (1), *E. coli* (5), *Bacillus subtilis* (6), and other microorganisms. This study necessitated the development of techniques for screening large populations of cells for such mutant types, and, in addition, the determination of the frequency of spontaneous occurrence of such mutations in the strain of *S. cerevisiae* employed.

MATERIALS AND METHODS

The strain used in these studies (referred to as "wild-type") was a haploid clone of *Saccharomyces cerevisiae* designated as strain 93-1C (α) kindly supplied by Dr. C. C. Lindegren, and orignially derived from a single ascospore.

Throughout these studies the synthetic medium of Burkholder *et al.* (7) was used, with the substitution of *Neurospora* trace elements (8) for those used by Burkholder. The "minimal" medium referred to hereafter is this basal medium supplemented with the following vitamins: biotin (10 γ/l.), pyridoxine, calcium pantothenate, *p*-aminobenzoic acid, inositol, and thiamine (1 mg. each/l.). Very good growth of the wild-type strain was obtained in 48 hr. at 30°C. The "complete" medium used consisted of 0.5% yeast extract (Difco), 0.3% peptone (Difco), and 1% glucose (c.p.). Both media were solidified by the addition of 1.5% agar (Difco) as desired.

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Growth was measured routinely by comparative visual estimates because this strain has a tendency to clump in liquid media. In certain instances, however, turbidity measurements were made with a Klett-Summerson photoelectric colorimeter to determine final growth, after the cells were autoclaved for 15 min. at 15 lb. to break up the clumps.

Treatments with the mutagen tris-(β-chloroethyl)amine (TBA) were carried out as follows: Dilutions of TBA hydrochloride[3] were made in sterile distilled water, the exact concentrations varying in different experiments (see Table I). Aliquots of cell suspensions in *M*/20 phosphate-citrate buffer (pH 5.9–6.0) were then added to the TBA hydrochloride solutions. After 10 min. the suspension was mixed with liquified agar at 45°C. and the mixture plated immediately. For the screening of large populations of both treated and untreated cells, use was made of a technique previously developed in this laboratory (9) and modified as follows: Cells were plated into minimal agar medium, incubated for 72 hr., and the "primary" colonies that appeared were marked and counted. The plates were then layered with a "complete" medium and incubated for an additional 48 hr. The low sugar concentration used (0.1%) prevented the primary colonies from growing excessively during this period. At the end of the second incubation period, "secondary" colonies appeared and were counted, isolated and tested. In control experiments with mixtures of known mutants and wild-type, good differentiation was obtained and recoveries of the original populations plated were satisfactory.

Subsequent and more extensive use of this method with both treated and untreated material has shown that between 20 and 40% of secondary colonies were mutants (see Table I, Col. 6), while the recovery of the population originally plated was better than 90%. For example, Col. 3 of Table I gives the fraction of the total population which appeared after layering with complete medium. Experiment 28b was made with seeded agar from experiment 28a, omitting only the complete medium layer. The primary colonies were marked at 72 hr., incubated, and secondaries counted 48 hr. later. By comparison of the figures from these two tests (Col. 3 and 7), it can be seen that not only was the number of secondaries more than doubled in the layered plates, but the recovery of mutants in 28a was nearly 3 times as great as in the unlayered control set. Following TBA treatment, the percentage of secondaries was greatly increased (compare Expt. 32 with Expts. 26, 27, and 28a, Table I, Col. 3). However, the proportion of mutants among these secondary colonies were approximately the same as that found for untreated cells.

To facilitate further the sampling of large populations for the presence of mutants, a plate-test method has been devised to replace the standard practice of isolating colonies, transferring them to tubes of complete medium and subsequently testing them in liquid minimal medium. The plate-test is carried out in a transfer room as follows: For each group of isolates to be tested, two plates of minimal agar and one of com-

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[3] Supplied through the courtesy of Dr. J. S. Fruton.

TABLE I

Comparison of Spontaneous and TBA-induced Mutation Frequency In a Strain of S. cerevisiae

Mutagen treatment	Expt. no.	1 Total no. colonies	2 Total number secondaries	3 Per cent total as secondaries	4 Number secondaries tested	5 Total number mutants	6 Per cent secondaries as mutants	7 Total per cent mutants per survivors
None	26	1116	48	4.3	34	9	26.5	1.1
None	27	4162	133	3.2	127	51	40.1	1.3
None	28a	1369	71	5.2	71	14	19.7	1.02
Totals or averages of 26, 27, and 28a		6647	252	3.8	232	74	31.9	1.2
TBA 0.03%	8	27000	—	—	—	—	—	—
TBA 0.10%		6744	—	—	35[a]	14[c]		6.6
TBA 0.30%		178	—	—	178			
TBA 0.04%	32	52000	—	—	—	—	—	—
TBA 0.06%		1911	1048	54.8	41	10	24.4	13.4
TBA 0.08%		328	219	66.8	122	19	15.6	10.4
TBA 0.10%		4	2	50.0	2	0	0	0
None[b]	28b	1446	35	2.4	35	5[d]	14.3	0.35

[a] Layering technique not used; numbers represent random isolations.

[b] Layering omitted.

[c] Requirements of mutants obtained: methionine (2), adenine (1, strain 99R), leucine plus lysine (2), methionine plus lysine (2), methionine plus niacin (1), methionine plus a partial requirement for phenylalanine (1, strain 46) and yeast-extract (5).

[d] Strains with partial requirements.

plete agar medium are prepared and allowed to dry overnight at room temperature. During the test the plates are marked with a reference point and rest on printed "grids" marked into 45 numbered squares which serve as guides for inoculation. Secondary colonies are isolated in the usual manner and each suspended in 2–4 drops of sterile distilled water contained in sterile Pyrex depression slides. A 1 mm. loop is then used to transfer drops consecutively to the minimal and complete media. The test plates are incubated 40–50 hr. at 30°C., after which time mutant and wild-type strains may be readily differentiated by their relative growth on the complete and minimal media. Mutants can then be transferred to slants of complete medium for subsequent testing.

Using this method, it is possible to test 45 isolates per set of three plates with a minimum of time, labor and materials. In addition, the technique makes possible the detection of unstable mutants which are distinguished from stable mutants by the development of papillae of reverted cells on the minimal plates. In the studies reported here, such unstable strains represented about 6–7% of the mutants and have been included in the calculations of mutation frequency, since they are easily identifiable as mutants.

Application of the plate-test method to the identification of the requirements of the mutants has not been completely successful. The auxanographic method of Beijerinck (10) has also been tried, but in general has given unsatisfactory results. The requirements reported in Table II were determined by growth in liquid media, but the procedure is slow and somewhat unsatisfactory. With either technique, strains with multiple requirements are difficult to identify.

TABLE II

"Absolute" and "Partial" Vitamin Requirements of Wild-type S. cerevisiae

	Vitamin omitted	Growth[a]		Mean
	All	20	21	20.5
Partial requirement	B_1	107	116	111.5
	B_6	118	101	109.5
	Inositol	69	84	76.5
Absolute requirement	PABA	26	24	25.0
	Ca pantothenate	21	29	25.0
	Biotin	74	75	74.5[b]
	None	199	182	190.5

[a] Klett readings on duplicate cultures; 48 hr. growth, 30°C.

[b] See text.

Spontaneous Mutation Frequency

As summarized in Table I, in a total of 6674 untreated cells screened, 1.2% were found to be nutritionally deficient. This frequency of spontaneous biochemical deficiencies is high in comparison with the frequencies found for *Neurospora crassa* (11) and *E. coli* (12). It is felt, however, that this value is approximately correct since, for Expts. 26 and 27, summarized in Table I, the wild-type strain was reisolated by serial plating on minimal medium and stock cultures were carried on solid minimal medium. Later, to provide comparable material for future work, the wild-type was again reisolated on minimal medium, and an entire colony distributed in several aliquots and lyophilized. Expts. 28 and 32 were performed with this material, removed from lyophil and grown for 72 hr. on minimal agar slants. These precautions would be expected to minimize the survival of mutant cells of spontaneous origin. It would seem probable therefore, that mutant types isolated from this untreated material actually represent products of spontaneous mutation during the period of the experiment.

Mutation Frequency After Treatment with TBA

After treatment with TBA, the cells were plated in complete medium, incubated for 48 hr., and random isolates tested in liquid minimal medium. As shown in Table I, Expt. 8, 6.6% of the survivors were biochemical mutants. Repetition of the mustard gas experiments, employing more accurate techniques and more rigid standards, yielded frequencies in excess of 10% (Table I, Expt. 32)—a 10-fold increase over the frequency of spontaneously-occurring biochemical deficiencies. On the basis of these increases in mutation rate, TBA appears to be a mutagen for the haploid strain of *S. cerevisiae* used in these experiments, as it has previously been shown to be for other organisms (2,5,11,13,14).

A comparison between the lethal action of the mustard gases and X-rays on *Drosophila* has shown certain similarities (12). Fig. 1 shows survival curves plotted from data obtained with yeast in the experiments with TBA just discussed. A straight-line survival-dose relation was found as for X-radiation of bacteria (15,16). This relation would be expected if both agents act at random on single sensitive targets. No explanation is apparent for the differences in slope of the two curves in Fig. 1. Conditions of treatment were identical; only the plating techniques differed and, on the basis of the evidence discussed above, the two procedures would have been expected to give similar results. However, the critical point for purposes of comparison with other agents and organisms is the shape of the curve, rather than its slope.

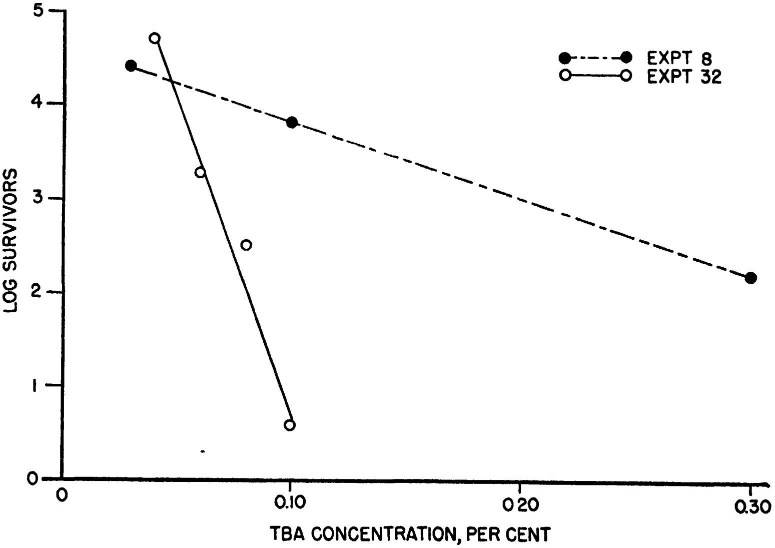

FIG. 1. Survival curves for *S. cerevisiae* strain 93-1C after treatment with TBA.

Characterization of Mutant Types

The requirements of 9 of the 14 mutant strains from Expt. 8 have been identified. These data are also summarized in Table I. It will be noted that single deficiencies were relatively rare, which is in contrast to the condition found in most other microorganisms which have been studied. Examination of over 70 additional biochemical mutants of this yeast has confirmed this finding. The addition of single amino acids, vitamins, or nitrogenous bases, did not result in growth responses comparable to wild-type growth rate. In most instances, nonspecific, graded growth responses to various supplements were obtained which rarely approached the wild-type rate. Since no genetic analyses are as yet available on these strains, no conclusions can be drawn as to the bases for such behavior.

The degree of dependence on growth factor supplements varies between strains and for different supplements with a given strain. A strain which will grow within 70 to 100 hr. in the absence of a given growth factor, but which will grow more rapidly in its presence, is arbitrarily defined as having a "partial" requirement. A strain in which growth is delayed past 100 hours under the same conditions, is classified as having an "absolute" requirement. In the former class are the inositol, B_6 and B_1 deficiencies of wild-type; the biotin, *p*-aminobenzoic acid and pantothenate requirements of wild-type exemplify the latter category. In Table II are given measurements of growth at 48 hr. for a series of cultures containing only 5 of the 6 vitamins at a time. In the absence of biotin, some growth was obtained in the initial culture, but serial transfer of cells from these cultures to a biotin-deficient medium did not result in continued proliferation. Hence, the requirement for biotin is considered to be a complete requirement; strains with partial deficiencies grow indefinitely, though slowly, in the absence of the specific supplement. The same range of differences in growth response were also found among the mutants listed in Table I.

The many *leucineless-lysineless* mutants recovered in Expt. 8 may not have been induced by the mustard gas treatment, but may have been present in the population at the time of treatment. Previous to this experiment, stock cultures had been carried on complete medium and the isolation of spontaneous mutants from one of these cultures yielded a very high percentage of strains which required leucine and lysine. By contrast, no *leucineless-lysineless* strains were found among

45 of the mutants from Expts. 26, 27, and 28a, for which the wild-type cultures had been reisolated and carried on minimal medium.

Strain 99R requires adenine and produces a pink pigment when grown aerobically on either minimal agar supplemented with adenine or on complete medium. On the latter medium, about 10% of the colonies were white and remained as stable white clones indefinitely on the standard 1% glucose medium; no pink clones have ever been reisolated from them under these conditions. However, these white derivatives of strain 99R formed pigment on media in which the glucose concentration had been raised to 8%. It was also found that a typical white variant (99W) required leucine and lysine in addition to retaining the original adenine deficiency (Table III), and that it was unable to utilize glycerol for aerobic growth, although both wild-type and 99R are able to do so. No pigment is formed by 99R growing on glycerol. These analyses are in a preliminary stage and no explanation of the results is available as yet.

TABLE III

Growth Factor Requirements of Strains 99R and 99W

Growth factor added	Growth of strain 99R[a]	Growth of strain 99W[a]
None	4.0	13.5
Leucine	7.5	23.0
Lysine	8.5	25.0
Leucine+lysine	11.0	42.5
Adenine	144.5	18.5
Adenine+leucine	144.0	39.0
Adenine+lysine	166.0	41.5
Adenine+leucine+lysine	133.5	147.5

[a] Cultures grown in minimal medium supplemented with the appropriate growth factors in a concentration of 0.01 mg./ml. Growth is expressed as the average of Klett readings on duplicate cultures. Strain 99R grown for 49 hr.; 99W for 73 hr., 30°C.

Two of the mutant strains have been subjected to a genetic analysis in Dr. C. C. Lindegren's laboratory (17). Both the methionineless (Strain 46) and adenineless (Strain 99R) characters segregated as single genes. It is presumed that the other strains reported here are likewise deficient biochemically as the result of mutation [for review see Lindegren (18)].

ACKNOWLEDGMENTS

The authors wish to thank Dr. and Mrs. Carl C. Lindegren for the wild-type haploid strain of *S. cerevisiae* and for the genetic data which they supplied; and Drs.

David Regnery and R. W. Barrett, Stanford University, for criticism of the manuscript.

Summary

A modification of the layer-plate technique for screening for nutritionally deficient cells of yeast, and a new plate-test method for testing prospective mutant strains, are described.

Using these methods a high frequency (1.2%) of spontaneously occurring biochemical mutant strains was found for a haploid strain of *Saccharomyces cerevisiae*. Treatment with tris-(β-chloroethyl)amine increased this mutation frequency by at least five- to ten-fold.

Most of the mutants obtained appeared to have more than one biochemical deficiency and the requirements of these strains for single amino acids, vitamins, purines or pyrimidines, were rarely absolute.

Since genetic analysis of a *methionineless* and of an *adenineless* strain has shown that these deficiencies are due to mutations of single genes, it seems reasonable to assume that the other nutritionally deficient strains described in this paper resulted from gene mutations.

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Chinese Tallow Nut Protein. II. Nutritive Value

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INTRODUCTION

The Chinese tallow nut (*Sapium sebiferum*) has been the subject of considerable interest due to its content of high quality drying oil. The nut also contains appreciable quantities of tallow and protein. If the industrial development of this product for the drying oil proceeds as is anticipated, then quantities of protein material will be available for economic disposal. The first publication of this series (1) gives a partial description of the protein fraction, its isolation and the amino acid and vitamin content. The present work was undertaken to determine whether any toxic substances are present in the tallow nut meal, and to confirm with feeding tests certain suspected amino acid and vitamin deficiencies based on the microbiological amino acid and vitamin analyses.

MATERIALS AND METHODS

The method for the preparation of the tallow nut meal used in the feeding tests has been described elsewhere (1). The essential steps in the procedure are: (a) extracting the tallow with hexane, (b) grinding to about 20 mesh, (c) extracting the oil with 99% isopropanol, (d) milling and (e) screening. The meal contained 42.6% protein based on the conversion factor of 5.7 which was derived from the 17.7% nitrogen content of the moisture- and ash-free protein (1). Other meals have been prepared by this method which contained 75% protein.

Albino rats 28–35 days of age were used as the experimental animals. Two males and 2 females in individual cages were used in each test, and each curve in the charts represents the average gain in weight of these 4 rats. The animals were weighed twice weekly. The composition of the basal ration is given in Table I. To compare the nutritive value of the tallow nut protein with proteins of a recognized nutritive value, 2 groups of rats were fed Ration 1, with the exception that the tallow nut meal was replaced with 10% egg albumin in one case and 20% casein in the other, with enough cerelose added to make 100%.

TABLE I

Composition of Basal Diets

	Ration 1	Ration 2	Ration 3	Ration 4
Tallow nut meal, g.	23.5	—	14	14
Casein (vitamin-free Labco) g.	—	—	—	14
Wheat flour, g.	—	70	77	—
Cerelose, g.	61.5	12	—	57
Salts,[a] g.	5	5	5	5
Wesson oil, g.	—	—	3	—
Lard, g.	9	9	—	9
Wood pulp, g.	—	3	—	—
Vitamins ADEK,[b] g.	1	1	1	1
Thiamin, HCl, mg.	0.4	0.4	0.4	—
Riboflavin, mg.	0.4	0.4	0.4	—
Pyridoxine, HCl, mg.	0.4	0.4	0.4	—
Ca pantothenate, mg.	2	2	2	—
Niacin, mg.	5	5	5	—
Choline chloride, mg.	200	200	200	—
Biotin, γ	10	10	10	—

[a] Richardson and Hogan (2).

[b] One g. of Wesson Oil containing: 3,000 I. U. Vitamin A, 425 I. U. Vitamin D, 2.5 mg. Menadione, and 2.5 mg. α-tocopherol acetate. Vitamins A and D were supplied as Mead Johnson's Oleum percomorphum.

RESULTS AND DISCUSSION

Amino Acid Deficiency in Tallow Nut Meal

It has been shown by microbiological assay (1) that the protein of tallow nut contained 1.6% methionine and 2.6% lysine. These data show that the protein would be low in both lysine and methionine for animal growth, and it seemed desirable to find out whether the protein would be adequate for growth if it was supplemented with these two amino acids. In carrying out this test, 4 groups of rats were given basal Ration 1 which contained 10% protein, and amino acid supplements as follows: Group 1 received no supplement, Group 2 received 0.3% of L-methionine as DL-methionine, Group 3 received 0.3% of L-lysine as DL-lysine, and Group 4 received both lysine and methionine. The amount of amino acids added was sufficient to increase the tallow nut protein to 5.6% lysine and 4.6% methionine. The results are given in Fig. 1.

The addition of methionine alone increased the rate of growth slightly, and lysine alone gave a more rapid rate of growth than methionine. When both amino acids were added tallow nut protein was equal to or slightly superior to egg albumin, a protein which is recognized to be of very high quality. These data show that tallow nut meal as a source of protein for the growth of rats is deficient only in lysine and methionine, and it does not contain any substances toxic to rats when it is fed at a level equivalent to 10% of protein.

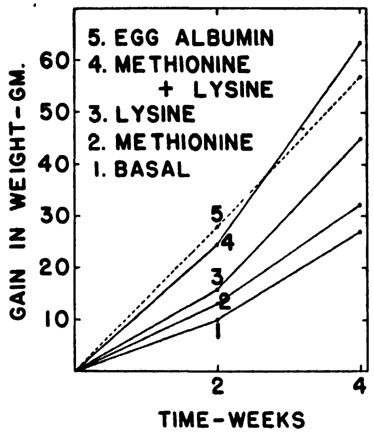

FIG. 1. Comparison of tallow nut meal, egg albumin, and tallow nut meal supplemented with amino acids as sources of protein for the growth of rats.

Tallow Nut Protein as a Supplement to Wheat Flour

Tallow nut flour (1) contains a high level of thiamine and it has been suggested that this material might be used for the enrichment of wheat flour with thiamine if it did not decrease the nutritive value of the protein of the enriched flour. Accordingly, tests were carried out first to compare the nutritive value of wheat flour with the above results for tallow nut meal, and, secondly, to determine how well the tallow nut meal would supplement wheat flour. In the first test, 2 groups of rats were fed Ration 2 (10% protein from wheat flour), with one group receiving the same lysine plus methionine supplement as described above. This comparison is given in Fig. 2.

The nutritive value of the tallow nut protein is definitely superior to the nutritive value of the wheat flour protein. This difference is much greater with the lysine-methionine supplements, indicating that the wheat flour lacks something besides these two amino acids. It is evident that the tallow nut meal would not decrease the nutritive value in a mixture of the two.

For the second test, additional groups of rats were fed Ration 3 with the same supplements of lysine and methionine as described in the preceeding section. This ration contained 17% protein (11% from wheat flour and 6% from tallow nut meal). The results are summarized in Fig. 3.

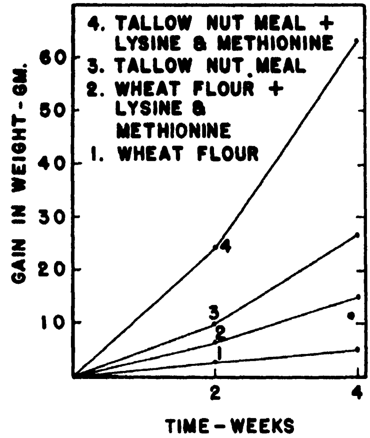

FIG. 2. Comparison of tallow nut meal and wheat flour as sources of protein for the growth of rats.

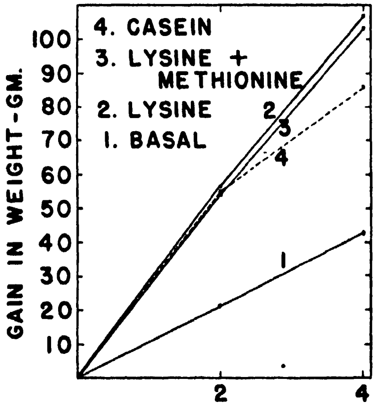

FIG. 3. Tallow nut meal and tallow nut meal plus amino acids as protein supplements to wheat flour.

The basal ration supported a very slow rate of growth, but when it was supplemented with lysine alone, the rats gained an average of 20 g. more than those which received 20% of casein. The mixture of wheat flour and tallow nut meal supplied an adequate amount of methionine when fed at the level used in this ration.

Vitamin Deficiency in Tallow Nut Meal

It was shown by microbiological assay (1) that the tallow nut contains relatively small quantities of riboflavin and pantothenic acid. Tests were carried out to determine whether or not sufficient quantities of the other B vitamins would be supplied for a normal rate of growth when the tallow nut meal furnished 6% of the protein in a diet that contained a total of 20% protein. These tests were carried out with 5 groups of rats which received basal Ration 4 and supplements/100 g. of diet as follows: Group 1 received no supplement, Group 2 received 2 mg. of calcium pantothenate, Group 3 received 0.4 mg. of riboflavin, Group 4 received 2 mg. of calcium pantothenate plus 0.4 mg. of riboflavin, and Group 5 received all the vitamins in the amounts listed in Table I, Ration 1.

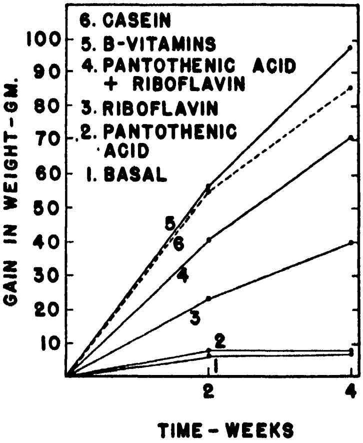

FIG. 4. Tallow nut meal as a source of B vitamins.

The average rate of gain of the rats which received these supplements and of those which received a similar diet that contained 20% of casein are given in Fig. 4. Those which received the basal diet and those which received the calcium pantothenate alone gained an average of 8 g. in 4 weeks. The average gain was increased to 40 g. by the addition of riboflavin alone, and to 70 g. by the addition of both vitamins. These data show that riboflavin was the first and pantothenic acid the second limiting vitamin in tallow nut meal. When the mixture of all the B vitamins was added, the rats gained faster than those which received the 20% casein diet. This indicates that at least one other vitamin is limiting for an optimal growth rate when all the B vitamins are supplied by tallow nut meal in an amount equivalent to 6% protein.

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SUMMARY

Chinese tallow nut meal was tested as a source of amino acids and as a source of B vitamins for the growth of rats. The meal as the sole source of protein in the diet contained an insufficient amount of lysine and of methionine to support a normal rate of growth, but when it was supplemented with lysine and methionine it was slightly superior to egg albumin.

Riboflavin is the first and pantothenic acid is the second limiting vitamin in tallow nut meal. Some other B vitamin or vitamins is present in insufficient amounts to support a normal rate of growth when fed in a diet at a level equivalent to 6% protein.

Tallow nut meal does not contain any substances toxic to rats when fed at a level equivalent to 10% protein.

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Changes in the Prothrombin Time Induced by Methylxanthines. Role of the Plasma Cofactor of Thromboplastin

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INTRODUCTION

In 1943, Topelberg and Honorato (1) demonstrated that caffeine can shorten the prothrombin time of chickens maintained on a vitamin K-deficient ration, but only temporarily and with a weaker action than vitamin K. Honorato, Ivanovic and Palma (2) found a similar action on the prothrombin time of rats on a low choline diet. Field *et al.* (3) subsequently studied the action of large doses of caffeine, theobromine and theophylline on dogs, rabbits and rats, using the one-stage method of Quick (4), but diluting the plasma to 12.5% with saline, and obtained a shortening of the prothrombin time for many days after these drugs were administered. Link and coworkers (3a) also indicated that the methyl xanthines augment the fibrinogen level above the normal ranges. Quick (5) was unable to repeat the latter experiments, but he diluted with fresh human plasma treated with $Al(OH)_3$. Scherf and Schlachman (6), working with plasmas diluted to 12.5% with saline, found that caffeine shortens the prothrombin time in man, but Blood and Patterson (6a) reported that aminophyllin had no significant effect on the prothrombin time in man. On the other hand, Deutsch and Gerarde (7) believe that the shortening of the prothrombin time found by Link's group is produced by the fibrinogen exclusively.

We have studied the same problem, particularly with respect to the role of the thromboplastin cofactor, the principle necessary for the conversion of prothrombin into thrombin, with thromboplastin, recently described by the author (8). This factor is adsorbed by $Al(OH)_3$ at a concentration of 0.2 M but is not adsorbed when the $Al(OH)_3$ is less concentrated than 0.05 M. Furthermore, any preparation of fibrinogen from rabbit or bovine will carry it as an impurity if special precautions are not taken. Thus, any dilution of plasma with human plasma treated with an insufficient amount of $Al(OH)_3$, or with fibrinogen without special treatment, will contain this thromboplastin cofactor (PCT).

More or less simultaneously, Owren (9) described a new factor, the factor V, and Seegers' group (10) the AC-globulin, both resembling PCT action. Nevertheless, we have not analyzed the PCT and thus we are not able to identify it with, or differentiate it from, these factors.

EXPERIMENTAL

Techniques

Caffeine (citrate), theobromine and theophylline were given to rabbits by stomach tube in single doses of 100 mg./kg. body weight. Sodium benzoate was used at the same dose level, also, as a control, because theobromine was dissolved in water with it.

To determine the prothrombin time, the one-stage method of Quick (4) was used with undiluted and diluted plasma. The thromboplastin agent used was dried rabbit brain. Dilutions of 20, 10, and 5% were made with distilled water and human and rabbit fresh plasmas treated with 0.01 M, 0.1 M and 0.2 M $Al(OH)_3$ (used as diluents).

The influence of fibrinogen on the prothrombin time was studied by making dilutions of plasma with human, rabbit, or bovine fibrinogen. The human and rabbit fibrinogen were prepared by treatment of plasma with sufficient NaCl to obtain 50% saturation. Bovine fibrinogen was prepared by the technique of Seegers (11).

TABLE I

Effect of 100 mg./kg. Body Weight of Caffeine, Theobromine, Theophylline and Sodium Benzoate in a Single Dose on the Prothrombin Times of Rabbits, Using 10% Diluted Plasmas

Rabbit	Substance used	Prothrombin time (sec.)						
		Before	After 1 day	2 days	3 days	4 days	5 days	7 days
1	Caffeine		14	8	10	9	—	—
2			14	8.5	11	11	10	15
3			15	10	11.5	10.5	11	15
4			16	10	9	11	11	12
Average		20±2	14.7±0.4	9.1±0.5	10.3±0.5	10.3±0.4	10.6±0.3	14±1
5	Theobromine		14	12	13	12	14	17
6			11	12	10	12	13.5	16
7			12	11	13	11	—	—
8			18	9	11	11	12	14.5
Average		20±2	13.7±3.7	11±1.2	11.7±3.9	11.5±0.5	13±1.2	15.8±1
9	Theophylline		14	10	8.5	9	14.5	
10			10	10	9	—	—	
11			17	9	13	15	17	
12			16	8	11	14	—	
Average		20±2	14±1.5	9.2±0.4	10.3±1.0	12.6±1.6	15.7±1.2	
13	Sodium benzoate	20±2	21	9	11	11	12	14
14 to 27	None	20±2						

RESULTS

Effect of Caffeine, Theobromine, Theophylline and Sodium Benzoate on the Prothrombin Time of Rabbits

The prothrombin time of undiluted rabbit plasma, with a value of 6 sec., was not changed by the administration of any of these compounds. Only thromboplastin, which gave 12 sec. with fresh human plasma, was used. The results are recorded in Tables I and II. The results obtained with 20% diluted plasmas gave no significant changes in comparison with untreated animals.

TABLE II

Effect of 100 mg./kg. Body Weight of Caffeine, Theobromine, Theophylline and Sodium Benzoate in a Single Dose on the Prothrombin Times of Rabbits Using 5% Diluted Plasmas

Rabbit	Substance used	Prothrombin time (sec.)						
		Before	After 1 day	2 days	3 days	4 days	5 days	7 days
1	Caffeine		25	14	30	30		
2			22	15	22	28	34	27
3			24	20	21	32	32	39
4			31	18	21	32	31	30
Average		36±1.7	25.5±1.9	16.7±1.3	23.5±2.1	30.5±1	32.3±1.1	32.5±5.1
5	Theobromine		30	24	30	27	29	39
6			17	20	20	22	27	37
7			23	23	29	22		
8			32	17	20	23	23	29
Average		36±1.7	25.5±3.4	21±1.4	24.7±2	23.5±1	26.3±1	35±3
9	Theophylline		50	26	17	23	38	
10			33	23	19.5			
11			40	20	26	25	111	
12			45	15	20	23		
Average		36±1.7	42±3.5	21±2.3	20.6±2	23.6±0.5		
13	Sodium benzoate	36±1.7	40	17	20	23	23	29
14 to 27	None	36±1.7						

Dilution of Normal Rabbit Plasma with Human and Rabbit Fresh Plasmas Treated with $Al(OH)_3$ in Increasing Concentrations

The plasmas used to dilute the rabbit plasma were prepared by treatment with $Al(OH)_3$ to obtain concentrations of 0.01 *M*, 0.1 *M* and 0.2 *M* (8). The importance of albumins left in the human plasma was studied by making dilutions of the rabbit plasma with human albumins, obtained by precipitation of human plasma with $(NH_4)_2SO_4$, and then dialyzing until all the albumin fraction was ammonia-free (12). The results are recorded in Table III.

TABLE III

Dilution of Normal Rabbit Plasma with Human and Rabbit Fresh Plasmas, Treated with $Al(OH)_3$, on Increasing Concentrations and Its Influence on the Prothrombin Time

	Prothrombin time (sec.)
Normal rabbit plasma diluted 5% with rabbit alumina plasma 0.01 *M*	19
Normal rabbit plasma diluted 5% with rabbit alumina plasma 0.13 *M*	26
Normal rabbit plasma diluted 5% with human alumina plasma 0.01 *M*	20
Normal rabbit plasma diluted 5% with human alumina plasma 0.1 *M*	35
Normal rabbit plasma diluted 5% with human alumina plasma 0.2 *M*	42
Normal rabbit plasma diluted 5% with human albumin fraction from plasma	41
Normal rabbit plasma diluted 5% with distilled water or saline	32

Prothrombin-Free Caffeine Plasma and its Influence on the Prothrombin Time of Normal Rabbit Plasma

The plasma obtained from rabbits receiving caffeine was treated with 0.04 *M* $Al(OH)_3$, thus eliminating the prothrombin but not the thromboplastin cofactor. This plasma was then diluted to 10% with distilled water and 0.02 cc. were added to 0.1 cc. of normal rabbit plasma diluted to 5% with water. The prothrombin time of this 0.12 cc. was determined by the usual method. As a control, identical experiments were performed, using animals that had not received caffeine. The results are recorded in Table IV.

TABLE IV

Prothrombin Time of Normal Rabbit Plasma Diluted 5% plus 0.02 cc. of Normal or Caffeine Prothrombin-Free Rabbit Plasma Diluted 10% or 20%. Distilled Water Used to Dilute

	Prothrombin time (sec.)
Normal plasma diluted 5% + 0.02 cc. normal alumina plasma dil. 20%	28
Normal plasma diluted 5% + 0.02 cc. caffeine alumina plasma dil. 20%	21
Normal plasma diluted 5% + 0.02 cc. normal alumina plasma dil. 10%	35.5
Normal plasma diluted 5% + 0.02 cc. caffeine alumina plasma dil. 10%	19
Normal plasma diluted 5%	29

It is evident that prothrombin-free plasma from animals receiving caffeine can reduce the prothrombin time of 5% plasma from normal animals. The observation must be made with diluted normal plasma and diluted caffeine plasma, because the normal concentration of the thromboplastin cofactor is very high in rabbits. The dilution allows the investigation of any increase in this factor.

Prothrombin Time of Normal Rabbit Plasma Diluted to 5% with Human, Rabbit or Bovine Fibrinogen

Human and rabbit fibrinogen were diluted with saline to give the same concentration as the original plasma. Bovine fibrinogen was concentrated from 100 cc. of plasma to 16 cc. of saline. Then rabbit plasma was diluted with the fibrinogen solution instead of saline, and the prothrombin time was determined. To study the real meaning of fibrinogen in the shortening of the prothrombin time, all the fibrinogen was eliminated from the fibrinogen solution. In the rabbit fibrinogen solution this was done with very small amounts of thrombin and then by heating to 60°C. for 10 min. to destroy the thrombin; in the bovine solution, the fibrinogen and the prothrombin were inactivated by heating to 58°C. The rabbit plasma was then diluted with these solutions. If the effect on the prothrombin time was maintained it would be the result of an impurity carried by the fibrinogen solution, but not the effect of fibrinogen itself. The results are recorded in Table V.

TABLE V

Prothrombin Time of Normal Rabbit Plasma Diluted with Human, Rabbit or Bovine Fibrinogen Solution

	Prothrombin time (sec.)
Normal plasma diluted 5% with rabbit fibrinogen solution	26
Normal plasma diluted 5% with rabbit fibrinogen solution after thrombin treatment and heating for 10 min. at 60°C.	23
Normal plasma diluted 5% with human fibrinogen solution	50
Normal plasma diluted 5% with bovine fibrinogen solution	15
Normal plasma diluted 5% with bovine fibrinogen solution heated to 58°C. and thus without fibrinogen and prothrombin	15
Normal plasma diluted 5% with distilled water or saline	35

DISCUSSION

The shortening of the prothrombin time of rabbit plasmas under the influence of a single dose of caffeine, theobromine, theophylline or sodium benzoate is evident (Tables I and II). At the same time it is clear that these results can only be observed with plasma diluted to 10 and 5%. The reason why some investigators obtained different results is easy to explain on studying Table III. If dilution of normal rabbit plasma is made with human fresh plasma (instead of water or saline) treated with 0.01 *M* $Al(OH)_3$, the prothrombin time will be shorter than it should be, because this treatment does not eliminate the thromboplastin cofactor. This factor will produce an important error, because the normal values will be around 20 sec. instead of 35 or 36 sec. for 5% diluted plasma, and, therefore, no changes will be observed under the influence of drugs. But if the concentrations of $Al(OH)_3$ are increased, the prothrombin times obtained are completely different; with 0.1 *M* the prothrombin time is 35 sec. and with 0.2 *M* it is 42 sec.

In any case, it is indispensable to use an alumina solution with molarity enough to take out all the PCT. We suggest first trying the prothrombin time on plasma diluted with saline and then on plasma diluted with plasma treated with $Al(OH)_3$ of different concentrations. The correct molarity of alumina to be used must be that which can produce diluted plasmas giving a prothrombin time similar to that given by the saline dilution.

We think that the explanation for the increase of the prothrombin time on diluting with plasma treated with 0.2 *M* $Al(OH)_3$ beyond that for the saline-diluted is the adsorption of the thromboplastin cofactor (from the caffeine plasma) by the albumin of the diluting human plasma. The results obtained by diluting with the albumin fraction of human plasma instead of using the whole plasma treated with $Al(OH)_3$ permit this hypothesis (Table III).

From this part of the work it can be established that:

1. Dilution of human plasma with an insufficient amount of $Al(OH)_3$ fails to remove the thromboplastin cofactor, thus producing an increase of this factor in rabbit plasma and giving abnormally short prothrombin times.
2. Treatment with 0.1 *M* $Al(OH)_3$ produces values similar to those obtained on diluting with distilled water or saline, and the difference between the normal plasmas and the plasmas from rabbits receiving drugs are very clear.

But, as was demonstrated by us in a previous paper (8), the prothrombin time is the result of two factors, at least: the prothrombin itself and the plasma thromboplastin cofactor. Therefore, any change in the prothrombin time can be the result of an increase or decrease of any of these factors. The experiments recorded in Table IV prove that the shortening of the prothrombin time obtained under the influence of caffeine, theobromine, theophylline or sodium benzoate are not due to increased prothrombin activity (or levels) because the plasmas from rabbits receiving caffeine treated with $Al(OH)_3$ and thus without prothrombin (diluted to 10% with water), are able to reduce the prothrombin time of normal rabbit plasma (diluted to 5% with water) from 36 sec. to 19 sec. The same experiment, carried out with plasma from normal animals not receiving caffeine, gave 33 sec.

Table V demonstrates that fibrinogen cannot be the substance that produces the shortening of the prothrombin time. This is due rather to another fraction carried with it, because the total elimination of fibrinogen does not change the results. The increase of fibrinogen observed on the animals receiving methylxanthines is a parallel phenomenon, but not the one responsible for the shortening of the prothrombin time.

From a study of this same table, it can be established that this factor, which we believe is the same one we have designated as the thromboplastin cofactor, is many times more concentrated in rabbit and bovine plasmas than in human plasma.

SUMMARY

1. The influence of caffeine, theobromine, theophylline and sodium benzoate on the prothrombin time was studied.

2. The ability of these substances to shorten the prothrombin time is demonstrated.

3. If dilution of rabbit plasma is made with human fresh plasma treated with $Al(OH)_3$, this treatment must be done with 0.1 *M* solution to be sure that all the thromboplastin cofactor is eliminated. Special precautions must also be taken if fibrinogen solutions are used as a diluent.

4. The shortening of the prothrombin time is not due to increase of the prothrombin activity (or level) and the results obtained when dilution is made with fibrinogen solution are due to an impurity carried by the fibrinogen and not to the fibrinogen itself.

5. It is believed that these results are produced by the increase of the thromboplastin cofactor and that bovine and rabbit blood contain several times the amount present in human blood.

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Quantitative Estimation of Some Amino Acids in Sweet Corn Pollen [1]

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INTRODUCTION

Considerable interest has been shown in recent years in the study of the chemical composition of pollen, especially that from corn. Pollen plays an important part in the process of fertilization and reproduction and as food for bees. Any knowledge pertaining to kind and quantity of chemical constituents present is of interest to the apiculturist as well as the plant physiologist. Although sufficient data on the percentage composition of pollens from different sources with respect to moisture, protein, fat, ash, and the various minerals and vitamins have become available as a result of several detailed studies (1–6), little is known about the amino acid composition. In a recent report by Auclair and Jamieson (7) the qualitative composition of the amino acids in pollen as collected by bees is given. The objective of this report is to provide quantitative data on composition of a portion of the amino acids found in freshly collected sweet corn pollen.

EXPERIMENTAL

Methods of Collecting and Preparing the Pollen for Analysis

The pollen was collected from Golden Cross Bantam sweet corn, grown under field conditions at the college farms during the summer of 1947. Tassels on which the anthers were beginning to dehisce were covered with No. 10 Kraft paper bags during the evening. The freshly shedding pollen was collected the following morning by shaking the tassels and removing the bags containing the pollen mixed with anthers. The anthers were separated from the pollen by screening and the cleaned pollen placed in cellophane bags, sealed, enclosed in waxed cartons, and stored in a deep freeze unit at a temperature of −20°C. The entire operation from the time the pollen was removed from the plants in the field until it was stored in the freezer required about 1 hr. The pollen was retained in a deep freeze unit until utilized for analysis.

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A sufficient quantity of the pollen was dried in the frozen state by sublimation. The resulting product was yellow and granular. This material, which contained 1.41% moisture, was used for subsequent analysis after being extracted with ether. The lipide-free material was kept in an amber colored bottle and preserved in a refrigerator until needed.

Methods

The amino acid determinations were carried out microbiologically using *Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides* as the test organisms. The media used in the various determinations were essentially the same as those described by Sauberlich and Baumann (8). Hydrolysis of the sample for assay was done according to the procedure adopted by Stokes *et al.* (9). Patton *et al.* (10) have found that there may be a considerable loss of lysine and arginine during acid hydrolysis in the presence of reducing sugars. Block and Bolling (11) have discussed possible losses of other amino acids during acid hydrolysis. For tryptophan, the method of Wooley and Sebrell (12) was followed. Methionine assay was carried out according to the method of Lyman *et al.*, using *Leuconostoc mesenteroides* P-60. For glutamic acid, the method of Lyman *et al.* (13) was used. Cystine was determined by the Sullivan reaction, as modified by Evans (14), using a Cenco photelometer with filters No. 3385 and 4304. Media for stock cultures and the inoculum were prepared according to Kuiken *et al.* (15). Triplicate determinations were made on the composite pollen samples and all values were checked to within ±5%.

RESULTS AND DISCUSSION

The protein content of the pollen was found to be 26.88% (N × 6.25) when expressed on the moisture-, ash-, and lipide-free basis. Table I shows the amino acid content of the corn pollen protein in comparison to the amino acid values obtained from the literature (11,16) for other corn proteins.

It is realized that the amino acid composition of corn germ, gluten, zein, and whole corn obtained from the literature represent values obtained by both chemical and microbiological methods. However, the values should serve to give an approximate idea at least of the relative amounts of the various amino acids present in the different protein constituents of corn.

The total quantity of the 12 amino acids estimated constitutes about 13% of the dry weight of the sample. These acids account for about 50% of the weight of the total protein in the pollen. Table I indicates that cystine is present in the lowest amount (calculated to 16 g. N).

The quantity of histidine, methionine and tryptophan is almost the same and is about 3 times that of cystine. The remaining amino acids vary from 3.5 to 6.0 g., while glutamic acid occurred to the extent of 9.1 g. This variation is a general characteristic of many proteins.

TABLE I

A Comparison of the Amino Acid Content of Corn Pollen Protein, Germ, Whole Corn, and Other Corn Proteins

(Calculated to 16 g. N)

Amino acid	Pollen	Germ[a]	Gluten[a]	Zein[a]	Whole corn[a]
	g	*g.*	*g.*	*g.*	*g.*
Arginine	4.7	8.1	3.1	1.6	4.8
Histidine	1.5	2.9	1.6	1.0	2.2
Lysine	5.7	5.8	0.8	0.0	2.0
Tryptophan	1.6	1.3	0.7	0.2	0.8
Phenylalanine	3.5	5.5	6.4	6.6	5.0
Cystine	0.6	1.8	1.1	1.0	1.5
Methionine	1.7	1.6	2.5	2.0	3.1
Threonine	4.6	4.7	4.1	2.5	3.7
Leucine	5.6	13.0	24.0	25.0	22.0
Isoleucine	4.7	4.0	5.0	5.0	4.0
Valine	6.0	6.0	5.0	3.0	5.0
Glutamic acid	9.1	—	24.5[b]	35.6[b]	—

[a] Data obtained from Block and Mitchell (16).

[b] Data obtained from Block and Bolling (11).

A survey of the data also indicates that the distribution of the amino acids in corn pollen proteins is rather uniform, in contrast to the amino acid distribution of the corn germ, gluten, zein, and whole corn. Corn pollen protein contains considerably less leucine, cystine and phenylalanine, and more tryptophan than do the other proteins shown in Table I. Gluten and zein are higher in glutamic acid than corn pollen. These data suggest some of the differences between proteins in various parts of the reproductive structures in corn.

SUMMARY

A quantitative estimation of 12 amino acids in the protein of freshly collected sweet corn pollen is reported. Their distribution in the pollen protein is compared with that of other reproductive tissues in the corn plant.

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The Effect of Hypophysectomy, Adrenocorticotropic Hormone and Adrenal Cortical Extract on the Glucose Uptake and Glycogen Synthesis by the Isolated Diaphragm with and without Insulin [1]

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INTRODUCTION

It is well established (1–5) that insulin causes an increase in the glucose utilization and glycogen formation in the diaphragm *in vitro.* Corkill and Nelson (4) found that, although the basal glucose utilization of diaphragms from rats treated with anterior pituitary extract (APE) was within normal limits, the activating effect of insulin was decreased by the APE treatment. Furthermore, the insulin stimulation of glycogen synthesis was completely inhibited by APE treatment. These results were taken to support the findings of Colowick *et al.* (6), who discovered that insulin serves only to release the muscle hexokinase reaction from an inhibition exercised by the anterior pituitary and adrenal. As pointed out by Stadie and Zapp (2), if the conclusion of Colowick *et al.* is correct, insulin should have no accelerating effect on the muscle hexokinase reaction in hypophysectomized animals.

When our investigations with the diaphram of hypophysectomized rats were in progress, two groups of workers (7,8) reported that hypophysectomy does not abolish the effect of insulin upon glucose utilization. Perlmutter, Krahl and their coworkers (7,8) were able to show that the rate of glucose uptake by the diaphragms of hypophysectomized rats was about the same as that of normal animals. It will be seen that the present study confirms this conclusion. In addition, glucose

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utilization and glycogen formation of isolated diaphragms from rats treated with the adrenocorticotropic hormone (ACTH) or adrenal cortical extract (ACE) were also investigated.

EXPERIMENTAL

Male rats (Long-Evans strain) 40 days of age were used. The animals were hypophysectomized by the parapharyngeal approach and employed 10 days later. All animals were allowed to eat the usual diet *ad libitum*. The adrenocorticotropic hormone was isolated from sheep pituitaries by the method previously described (9), and was administered intraperitoneally 24 hr. before sacrifice. Adrenal cortical extract was a commercial preparation[2] (Upjohn), 1 cc. of which is derived from 40 g. of beef adrenals.

The experiments were carried out in two sets of conditions. In Series I, only glucose utilization was determined, and the procedure was similar to that employed by Krahl and Cori (3). The animals were killed by decapitation; the excised diaphragm was immediately placed in ice-cold Krebs-Henseleit solution having been cut into 6 approximately equal portions. Each piece of the tissue was incubated individually in a Warburg vessel. The vessels were shaken for 2 hr. in a bath at 37.4°C. The glucose concentration in the medium was 100 mg.-% and the volume of the medium was 0.3 cc. When insulin[3] (Iletin) was used, this was added to the medium in such amounts as to result in a final concentration of 1 unit/cc.

In Series II, both glucose utilization and glycogen synthesis were determined. The rats were killed by decapitation and the two lateral portions of the diaphragm were excised, blotted on filter paper and weighed on a torsion balance. One portion was immediately placed in 1.0 cc. of 30% KOH for the determination of the initial glycogen content. The other portion was placed in 2.0 cc. of ice-cold Krebs-Ringer-bicarbonate solution containing 100 mg.-% glucose in a Warburg vessel. The vessel was then joined to the manometer, placed in the thermostat (37.4°C.) and gassed with a mixture of 5% CO_2-95% O_2 for 15 min. After 2 hr. incubation, the vessels were disjointed and placed in ice-water. The tissues were removed, blotted and placed in 1.0 cc. of 30% KOH for the determination of the final glycogen content. The glycogen was determined by the method of Good *et al.* (10), the glucose resulting from hydrolysis being estimated by the Nelson method (11).

After removal of the tissues from the Warburg vessel, 0.10 cc. of the medium was deproteinized by the $Ba(OH)_2$-$ZnSO_4$ method and glucose was determined by the method of Nelson (11). Two determinations were carried out in each vessel. Glucose utilization was calculated by subtraction of the final glucose concentration from the concentration of glucose in the medium at the start of the incubation. The latter concentration was determined in a similar manner concurrently with each run. Glucose utilization and glycogen synthesis are expressed as mg. glucose/g. of wet tissue/hr.

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[2] Kindly furnished by Dr. D. J. Ingle.

[3] Whether the insulin preparation contains hyperglycemic factor has not been determined.

RESULTS

Glucose Uptake. Glucose uptake of the diaphragms of normal and hypophysectomized rats in Series I are summarized in Table I. It may be seen that the mean glucose uptake in the case of diaphragms from normal rats was 1.69 mg. without insulin and 2.17 mg. with insulin, and under the same conditions the uptake in diaphragms from hypophysectomized animals was 1.85 mg. without insulin and 2.24 mg. with insulin. In both cases, the accelerating effect of insulin was highly significant.

TABLE I

Glucose Utilization by Diaphragms of Normal and Hypophysectomized Rats

Six animals were used in each experiment; each diaphragm was cut into 6 pieces and each piece was run separately. Each value was, therefore, an average of 36 observations. The glucose uptake was expressed as mg. of glucose/g. of wet tissue.

Type of rats	Glucose uptake		P^b
	Without insulin	With insulin	
Normal	1.69±0.21[a]	2.17±0.21	<0.001
Hypophysectomized	1.85±0.17	2.24±0.25	<0.010

[a] Mean ± standard error.

[b] Fisher's P values, for significance of insulin stimulation.

Glycogenesis. Tables II and III present the data obtained with normal and hypophysectomized rats from Series II experiments in which glucose uptake and glycogenesis were determined in the presence and absence of insulin. In normal rats, the mean amount of glucose uptake by the diaphragm without insulin was 2.23 mg., and 3.78 mg. with insulin. The values for the diaphragms of hypophysectomized animals were 2.14 mg. without insulin, and 4.08 mg. with insulin. It may be noted that these values are considerably higher than those obtained in the Series I experiments. The cause for the deviation is not clear; it could be due to slight differences in the experimental conditions. It is apparent, however, that hypophysectomy does not alter the rate of glucose uptake by the diaphragm. As shown in Tables II and III, glycogenesis also does not differ significantly in normal and hypophysectomized rats. The glycogen formation in the absence of insulin was 0.22 mg. and 0.52 mg. for normal and hypophysectomized rats, respectively; with insulin, the values were 1.52 mg. and 1.66 mg.

Glucose Uptake and Glycogenesis in the Diaphragms of Normal Rats Treated with Adrenocorticotropic Hormone and with Adrenal Cortical Extract

A total of 18 normal rats was injected with 5 mg. adrenocorticotropic hormone intraperitoneally 24 hr. before sacrifice. Six of them were employed for glucose uptake and glycogenesis determinations without insulin and the remainder for similar determinations with insulin. As shown in Table IV, the mean amount of glucose uptake by the diaphragm from treated animals was 2.43 mg. in the absence of insulin and

TABLE II

Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats

Each value was obtained from the diaphragm of one rat, *i. e.*, one observation from one animal. The glucose uptake and glycogenesis are expressed as mg. of glucose/g. of wet tissue.

Rat number	Glucose uptake		Initial glycogen content	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
1	2.96	4.19	1.72	0.87	2.17
2	2.53	3.80	2.45	0.40	1.23
3	3.03	3.01	4.05	−0.11	1.07
4	2.49	2.65	4.23	−0.17	0.77
5	2.61	3.21	3.54	0.02	0.71
6	2.13	2.45	1.92	0.47	1.55
7	1.47	4.31	4.84	0.18	0.94
8	2.05	5.98	1.41	1.11	2.26
9	1.58	4.57	5.96	−0.21	2.42
10	2.16	4.54	4.15	−0.02	2.12
11	0.66		7.79	−0.46	
12	1.67		3.35	0.65	
13	2.78		5.14	0.03	
14	2.76		4.73	0.17	
15	1.76		2.31	0.53	
16	2.40		2.73	0.74	
17	1.91		5.40	−0.01	
18	2.72		5.02	0.22	
19	2.60		4.60	−0.05	
20	2.42		3.74	−0.02	
Mean..........	2.23	3.78	3.95	0.22	1.52
Standard error..	±0.13	±0.37	±0.36	±0.09	±0.21

TABLE III

Glucose Uptake and Glycogenesis by Diaphragms of Hypophysectomized Rats

Each value was obtained from the diaphragm of one rat; *i. e.*, one observation from one animal. The glucose uptake and glycogenesis are expressed as mg. of glucose/g. of wet tissue.

Rat number	Glucose uptake		Initial glycogen content	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
1	2.19	4.94	3.91	0.29	1.29
2	2.24	4.67	3.40	0.08	0.47
3	2.32	3.60	2.60	0.63	1.61
4	2.45	3.93	3.60	0.27	1.73
5	2.18	3.75	2.98	0.90	1.70
6	2.77	3.71	4.52	0.48	2.01
7	2.79	4.06	2.98	1.03	2.10
8	2.71	4.01	2.98	1.01	2.40
9	2.19		2.56	0.75	1.69
10	1.40		5.17	0.28	1.16
11	1.97		3.35	0.55	
12	2.14		5.34	−0.13	
13	0.84		1.52	1.53	
14	1.70		5.54	0.12	
15			6.23	0.31	
16			7.11	0.16	
Mean...... ...	2.14	4.08	3.99	0.52	1.66
Standard error..	±0.14	±0.17	±0.40	±0.11	±0.17

3.61 mg. with insulin. These values were almost identical with those obtained with the diaphragms of untreated animals. The glycogenesis was negligible without insulin and only 0.54 mg. with insulin. When statistical comparison was made between the glycogen deposition values in the presence of insulin in normal rats and rats treated with adrenocorticotropic hormone, a P value of < 0.01 was obtained, indicating that a significant inhibition of the insulin effect on glycogenesis is caused by the hormone treatment.

To extend this established inhibiting effect[4] of adrenocorticotropic hormone upon glycogenesis in the isolated diaphragm, normal rats were treated with adrenal cortical extract 24 hr. before sacrifice. Each rat received 3 cc. of the extract in 3 divided dosages during the 24-hr. period. Results, summarized in Table V, show that the adrenal cortical extract definitely caused glycogenolysis and that the insulin effect on glycogenesis was decreased.

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[4] The *specificity* of this inhibiting effect is evident from experiments employed iodinated preparation. It was found that the iodinated adrenocorticotropic hormone did not cause inhibiting effect upon glycogenesis in the ioslated diaphragm. As shown previously, the adrenocorticotropic activity of the hormone is abolished by iodination [Li, Simpson and Evans, *Arch. Biochem.* **9**, 259 (1946)].

TABLE IV

Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats Treated with Adrenocorticotropic Hormone

Each value was obtained from the diaphragm of one rat, *i. e.*, one observation from one animal. The animals were injected with 5 mg. of the hormone 24 hr. before sacrifice. The glucose uptake and glycogenesis are expressed as mg. of glucose/g. of wet tissue.

Rat number	Glucose uptake		Initial glycogen content	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
1	2.29		3.49	−0.01	
2	2.40		3.27	−0.43	
3	2.83		2.70	−0.80	
4	2.33		2.72	0.27	
5	1.93		6.20	−0.38	
6	2.82		4.39	0.21	
7		4.11	2.32		1.41
8		3.36	2.93		1.11
9		3.90	3.56		0.94
10		3.24	2.64		−0.26
11		3.84	2.80		−0.50
12		3.69	4.18		−0.97
13		3.19	2.08		−0.33
14		3.47	3.62		0.90
15		3.70	2.82		1.36
16		2.99	2.66		0.85
17		3.77	4.72		0.96
18		4.11	5.28		1.04
Mean..........	2.43	3.61	3.55	−0.19	0.54
Standard error..	±0.14	±0.10	±0.26	±0.17	±0.24

TABLE V

Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats Treated with Adrenal Cortical Extract

Each value was obtained from the diaphragm of one rat; *i. e.*, one observation from one animal. The animals were treated with 3 cc. adrenal cortical extract (Upjohn) 24 hr. before sacrifice. The glucose uptake and glycogenesis are expressed as mg. of glucose/g. of wet tissue.

Rat number	Glucose uptake		Initial glycogen content	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
1	2.52		4.49	−0.05	
2	2.48		2.11	0.19	
3	4.35		3.59	−1.31	
4	2.73		4.91	−0.26	
5	2.67		3.05	0.14	
6	1.96		4.74	−0.84	
7	1.87		4.43	−1.59	
8	1.50		7.82	−1.34	
9	1.46		5.52	−0.59	
10	1.46		5.70	−0.68	
11	1.99		4.85	−1.79	
12	0.99		6.82	−0.90	
13		3.12	5.26		0.25
14		3.20	3.76		0.84
15		3.24	5.35		0.61
16		2.97	5.57		0.36
17		3.06	3.86		0.79
18		2.84	6.32		0.31
19		2.20	4.76		−1.52
20		3.17	5.00		0.18
21		4.05	4.86		0.89
22		3.32	4.54		0.60
23		3.07	4.98		0.28
24		3.17	4.24		0.86
Mean........	2.15	3.12	4.88	−0.75	0.37
Standard error..	±0.26	±0.12	±0.24	±0.19	±0.19

DISCUSSION

The fact that the rate of glucose uptake by the isolated diaphragm of hypophysectomized rats with or without insulin does not differ from the rate found in the diaphragms of normal animals confirms the observations of other investigators (7,8). Since the rate of glucose uptake depends upon the activity of the hexokinase system, it may be said that the enzyme is not under the control of the pituitary or that the factors in the pituitary which lessen or increase the hexokinase reaction are present to the same extent and thus cancel each other.

According to Colowick *et al.* (6), we should not expect a stimulating effect of insulin *in vitro* upon glycogen formation in the hypohpysectomized rat diaphragm. It may be that the action of insulin is to release the inhibition of the hexokinase reaction by substances other than those emanating from the pituitary.

It is of interest to note that, although hypophysectomy does not decrease the ability of the isolated diaphragm to uitlize glucose, the action of insulin in promoting glycogen formation is greatly imparied in the diaphragms of rats previously treated with anterior pituitary extract as shown earlier by Nelson *et al.* (4). Our data, obtained with the diaphragms of ACTH-treated animals, show that the rate of glucose uptake with and without insulin remains unchanged but that the insulin effect on glycogenesis is decreased. In the absence of insulin, glycogenolysis actually occurred in the diaphragms of the treated rats. These results

TABLE VI

A Summary on the Glucose Uptake and Glycogenesis by Diaphragms of Rats Treated with Adrenocorticotropic Hormone (ACTH) or Adrenal Cortical Extract (ACE)

Experiment	Glucose uptake		Initial glycogen	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
Control	2.23±0.13[b] (20)[c]	3.78±0.37 (10)	3.95±0.36 (20)	0.22±0.09 (20)	1.52±0.21 (10)
ACTH	2.43±0.14 (6)	3.61±0.10 (12)	3.55±0.26 (18) P[a] $=0.3$	−0.19±0.17 (6) $P=0.05$	0.54±0.24 (12) $P<0.01$
ACE	2.15±0.26 (12)	3.12±0.12 (12)	4.86±0.24 (24) $P=0.05$	−0.75±0.19 (12) $P<0.001$	0.37±0.19 (12) $P<0.001$

[a] Each P value quoted is a measure of the statistical significance of the difference of mean in question as compared to the respective mean of the normal control. A P value of < 0.05 is taken to indicate a statistically significant difference.

[b] Mean ± standard error.

[c] Number in parenthesis indicates number of rats.

are confirmed and extended in the experiments using adrenal cortical extract. In Table VI a summary of the data for ACTH and ACE experiments is presented for comparison. It is evident that the insulin effect was not completely abolished in either case and, in fact, insulin accelerated glycogen synthesis in the diaphragms of rats treated with ACE to almost the same extent as in normal animals. In the experiments with ACTH, the amount of glycogen formed was about half that of the controls under the influence of insulin.

The observation that there is a breakdown of glycogen in the diaphragms of rats treated with ACTH or ACE is in harmony with the reports of Verzar and Wenner (5,12), who found that cortical steroids caused glycogen breakdown and decreased glycogen synthesis in the intact diaphragm of the rat *in vitro*.

SUMMARY

1. The action of insulin on glucose utilization and glycogen synthesis in the isolated diaphragm from normal and hypophysectomized rats and in rats previously treated with adrenocorticotropic hormone or adrenal cortical extract has been investigated.

2. The diaphragm of hypophysectomized rats utilizes glucose and synthesizes glycogen to the same extent as does that of normal animals.

3. There is no change in the glucose uptake of the diaphragm of rats injected with adrenocorticotropic hormone or with adrenal cortical extract, but glycogen breakdown occurs in these diaphragms.

4. The insulin effect in promoting glycogen storage is impaired in the diaphragms of animals treated with either adrenocorticotropic hormone or adrenal cortical extract.

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Inhibition of Enzymatic Proteolysis. II. Effect of Carbonyl Group Reagents on the Activity of Kidney Cathepsins [1]

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INTRODUCTION

A previous communication from this laboratory (1) described observations on the inhibitory effect of carbonyl group reagents on the activity of pepsin, trypsin, and papain and gave a more detailed account of the inhibition of pepsin by hydrazine. The present report deals with the effect of several carbonyl group reagents on the activity of the intracellular cathepsins from hog kidney.

Carbonyl group reagents as inhibitors for catheptic activity have not been studied in the past, with the exception of a report by Bergmann *et al.* (2), who found that phenylhydrazine in a concentration of 10^{-2} M/l. strongly inhibited the hydrolysis of carbobenzoxy-L-leucylglycylglycine by a crude cysteine-activated liver cathepsin preparation. The inhibition was apparently due to an effect of phenylhydrazine on the enzyme now classified as cathepsin II.

EXPERIMENTAL

1. Determination of Catheptic Activity

The synthesis of the substrates and the incubation experiments were carried out as described by Bergmann and coworkers (3). Carbobenzoxy-L-glutamyl-L-tyrosine served as substrate for the determination of cathepsins I and IV. No activator was added to the incubation mixture in the assay for cathepsin I. To elicit the activity of cathepsin IV, the presence of cysteine was necessary and the resulting hydrolysis was then due to the combined action of cathepsins I and IV. The synthetic substrate used for cathepsin II was benzoyl-L-arginine amide and for cathepsin III L-leucine amide. For the activation of cathepsins II, III, and IV, cysteine was added to the enzyme solution and the mixture was incubated for 2 hr. at 37°C. before addition of the substrate. The course of the enzymic hydrolysis of the substrate by cathepsins I and IV was followed by the titrimetric ninhydrin method (4). Silicone stopcock grease was found preferable to glycerol as lubricant for the glass joints of the reaction vessels in the ninhydrin procedure. The activities of cathepsin II and III were followed by measuring the liberated carboxyl groups with the microtitration method of Grassmann and Heyde (5). In all incubation mixtures the substrate concentration was 0.05 millimole/ml. and the solutions were buffered to pH 5.0 by citrate buffer, the final concentration being 0.125 *M* for cathepsins I and IV and 0.040 *M* for cathepsins II and III. All incubations were carried out at 37°C. in an accurately controlled water bath. Nitrogen determinations were performed according to a micro Kjeldahl method described by Clark (6). For the determination of nonprotein nitrogen, filtrates were prepared after precipitation of the proteins in 5% trichloroacetic acid solution. Enzymatic activities were numerically characterized by monomolecular reaction constants calculated from the hydrolysis data as $k = 1/t \times \log (a/a-x)$, where $a = 100$ and x is the per cent hydrolysis at the time t. Yields are expressed as k/g. fresh kidney/ml. incubation mixture and purity as C (proteolytic coefficient), which is k/mg. protein nitrogen/ml. test solution (7). The monomolecular constants given in the following tables are usually the averages of 3 values obtained from samples drawn after 120, 180 and 300 min. incubation time.

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2. Preparation of Hog Kidney Extracts

Preliminary experiments were carried out to compare the suitability of acetone-dried kidney powders with fresh kidneys as starting material for the preparation of extracts rich in cathepsins. Fresh kidney dry powder was prepared and extracted with NaCl solution as described by Schales (8). The crude extracts showed good activities for all 4 cathepsins and no significant loss occurred when part of the protein contained in these solutions was precipitated by adjustment of the pH to 5.0 with acetic acid and removed by filtration. However, dry powders which had been stored for several months, as well as various commercial kidney dry powders, yielded extracts containing practically no cathepsin III, and the cathepsin II activity was only about one-half of that observed in extracts from fresh kidneys or from freshly prepared kidney powders. Extracts from fresh kidneys were, therefore, used in all subsequent experiments.

Fresh swine kidneys were cleaned from fat and ground in a Waring Blendor with 2 l. of ice-cold distilled water/kg. kidneys. After standing overnight at 5°C. the mixture was centrifuged for 25 min. (4500 r.p.m., International Centrifuge Size 2, conical head). The supernatant solution was decanted and filtered (Extract A), cooled to 5°C. and brought to pH 5.0 by the slow addition of H_2SO_4 under rapid stirring. The mixture was left overnight at 5°C., centrifuged, and the precipitate discarded. The supernatant solution (Extract B) was fractionated with $(NH_4)_2SO_4$ at pH 4.9–5.0 and the cathepsins were found in the fraction soluble in 1.03 *M* (25% saturated) and precipitated by 2.05 *M* (50% saturated) $(NH_4)_2SO_4$. This fraction was isolated by filtration of the reaction mixture through a thin layer of Hyflo Super Cel, dissolved in distilled water and filtered to remove the filter aid. The clear brown solution (Extract C) was again treated with $(NH_4)_2SO_4$ and the fraction soluble in 1.03 *M* and precipitated by 2.05 *M* $(NH_4)_2SO_4$ was collected as before. This material was dissolved in water and, after adjustment of the pH to 7.0, dialyzed against 1% NaCl solution at 5°C. for 24 hr. and filtered (Extract D). In these fractionation experiments, $(NH_4)_2SO_4$ was added slowly as the solid salt under stirring and its concentration in the solutions was controlled by the titration of steam distillates from alkalinized samples. Sufficient time, usually standing overnight at 5°C., was allowed for the completion of the salt precipitations. Storage of Extract D in the frozen state in a dry ice chest did not lead to any appreciable loss in activity over a period of several months. Repeated thawing and freezing, however, caused losses, especially of cathepsins II and III.

During the course of activation experiments by incubation with cysteine it was noticed that a heavy precipitate settled out. The removal of this precipitate by filtration did not affect the catheptic activities, so that incubation with cysteine was used as a further step in the purification of these enzymes. Example: Cysteine (25 millimole/l.) was added to Extract C and the mixture was kept for 24 hr. at 37°C. and pH 5.0, filtered, and dialyzed against 1% NaCl solution for 24 hr. at 5°C. and pH 5.0. Incubation tests with the dialyzed material (Extract E) gave the results listed in Table I, which contains also the activities of the various other fractions. The marked decrease in the cathepsin III activity after the cysteine treatment was due to the dialysis and not to the incubation with cysteine. After this incubation, but before dialysis, cathepsin III gave a value of 9.3 for $k \times 10^4$/g. kidney/ml. Dialysis lowered this value to 2.5, corresponding to a loss of 73%.

TABLE I

Activity of Cathepsins at Various Purification Stages of Hog Kidney Extracts

Extract	Yield ($k \times 10^4$/g. kidney/ml.) Cathepsins				Purity (proteolytic coefficient$\times 10^3$) Cathepsins			
	I	II	III	I+IV	I	II	III	I+IV
A	23	17	14	30	—	—	—	—
B	24	18–25	10–14	32	1.0	0.5–1.0	0.3–0.6	1.3
C	—	25	15	32	—	2.4	1.4	3.1
D	6	17–23	7–9	28–29	1.2	3.5–3.6	1.1–1.8	4.4–6.0
E	4	30	2.5	26	1.3	10.0	0.8	8.6

(Range limits include the results of several independent preparations. Cysteine concentration, when used as activator, 0.015 millimole/ml.)

3. *Inhibition of Catheptic Activity by Carbonyl Group Reagents*

Parallel determinations were performed with aliquots of the same enzyme preparation in presence and absence of inhibitor. The titration values were corrected (for increases in acidity occurring in absence of synthetic peptides) as usual with the aid of controls incubated without the addition of substrate. The final concentration of enzyme, activator, buffer and substrate in the incubation mixtures were kept the same in the corresponding experiments with and without inhibitor. In tests with tyrosine standard solutions it was ascertained that the presence of phenylhydrazine (40 millimole/l.) and of hydrazine (200 millimole/l.) did not interfere with the ninhydrin assay method.

A survey of the effect of several carbonyl group reagents is given in Table II. Listed there are typical values selected from a number of experiments. Occasionally, deviations were observed, such as one-half as much inhibition with the same or a larger quantity of inhibitor. In following up these deviations it was found that the cathepsin and cysteine concentrations have a considerable influence on the degree of inhibition resulting from a given inhibitor concentration, as will be shown below.

In addition to the results given in Table II, it was found that dimedon (6 millimole/l.) and semicarbazide (12 millimole/l.) did not inhibit any of the 4 cathepsins.

TABLE II

Inhibition of Kidney Cathepsins by Carbonyl Group Reagents

Inhibitor	Concentration millimole/l.	Per cent inhibition of cathepsin			
		I	II	III	I+IV
Phenylhydrazine	20	31	89	100	31
	8	25	55	100	23
	4	19	47	100	17
	2	15	30	63	17
Hydrazine	200	9–18	42	61	—
	100	—	20	39	—
	50	—	8	—	—
	10	—	7	—	—
Hydroxylamine	26	0	10	100	0
	20	0	7	100	0
	2	0	0	84	0
	0.4	—	—	77	—
	0.2	—	—	42	—

The following cysteine concentrations (millimole/l.) were used: Phenylhydrazine tests: Cathepsin II 20, III 15, I + IV 12; hydrazine tests: Cathepsin II 20 (10 in experiment with 200 millimole hydrazine), III 10; hydroxylamine tests, 15 in all experiments. The enzyme activities in absence of inhibitor were as follows (expressed as $k \times 10^4$): phenylhydrazine tests, Cathepsin I 9.1–10.3, II 7.6–8.9, III 5.1–5.7, I + IV 14.9; hydrazine tests, Cathepsin I 9.2–10.7, II 11.9–22.8, III 19.1; hydroxylamine tests, Cathepsin II 5.1, III 5.8–7.2 and I + IV 13.9.

Dialysis of enzyme solutions, which had been incubated with phenylhydrazine for 2 hr. at pH 5.0 and 37°C., against 100 vol. of 1% NaCl solution at 5°C. for 24 hr. led only to slight increases in activity as compared with the undialyzed solutions. The following figures illustrate the extent of reactivation: Cathepsin I (incubated with phenylhydrazine, 50 millimole/l.; reduced to 30 millimole/l. on addition of substrate) before dialysis 61% inhibition, after dialysis 52% inhibition; Cathepsin III (incubated with phenylhydrazine, 8 millimole/l.; reduced to 4.5 millimole/l. on addition of substrate) before dialysis 72%, after dialysis 59% inhibition. These data are corrected for changes in activity resulting from the dialysis of the same enzyme solutions in absence of inhibitor. Such a correction was of particular importance for cathepsin III, which lost a considerable portion of its activity on dialysis.

4. Effect of Cathepsin Concentration on Inhibition

It was noted that the degree of inhibition caused by a given inhibitor concentration was not always the same when various cathepsin preparations were compared. Experiments were therefore set up in which the phenylhydrazine and cysteine concentrations were kept constant while the amount of enzyme in 5 ml. incubation mixture was varied by using equal volumes of various dilutions of a concentrated enzyme stock solution. From the results, shown in Table III, it appears that the relative enzyme concentration has a significant influence on the degree of inhibition caused by a given inhibitor concentration.

TABLE III

Effect of Enzyme Concentration on the Inhibition of Cathepsins by Phenylhydrazine

Cathepsin	Enzyme activity $k \times 10^4$ (without inhibitor)	Per cent inhibition (phenylhydrazine, 10 millimole/l.)
II	15.0	30
	8.2	61
	4.5	67
I+IV	36.4	4
	16.6	35
	8.1	44

(Cysteine concentration: Cathepsin II, 0.015, Cathepsin I + IV, 0.012 millimole/ml.)

5. *Influence of Cysteine Concentration on Inhibition*

There were indications that the degree of inhibition depended to some extent also on the concentration of the activator. Experiments were therefore performed in which the enzyme and the phenylhydrazine concentrations were kept constant and the cysteine concentration was varied. The results in Table IV show that the cysteine concentration has a noticeable effect on cathepsin II activity in absence of inhibitor and a considerable influence on the degree of inhibition caused by a given phenylhydrazine concentration. The anti-inhibitor effect of cysteine was not as marked when an enzyme preparation of a higher degree of purity was employed (Extract E) and led only to an initial increase in activity, which subsided as the incubation was continued.

TABLE IV

Influence of Cysteine Concentration on the Inhibition of Cathepsin II by Phenylhydrazine

Extract	Cysteine millimole/l.	$k \times 10^4$ (without inhibitor)	Per cent inhibition (Phenylhydrazine, 6 millimole/l.)		
			2	3	4.5 hr. hydrolysis
D	7.5	8.1	83	84	—
	15	8.9	67	65	—
	30	9.1	28	32	—
E	7.5	4.7	74	81	81
	15	4.6	71	82	74
	30	6.1	34	52	66

6. *Effect of Guanidine on Catheptic Activity*

De Ritis and Zacco reported recently (9) that guanidine hydrochloride inhibited the catheptic hydrolysis of fresh guinea pig liver. A guanidine concentration of 90–135 millimole/l. inhibited proteolysis by 74–76% during an incubation time of 8 hr. On the basis of this report, the effect of guanidine on the activity of kidney cathepsins was investigated. Guanidine was added (1 mole/l.) to Extract D and the mixture was incubated for 2 hr. at pH 5.0 and 37°C. A heavy precipitate formed and was removed by filtration. The filtrate had the same cathepsin I and IV activities as the extract before the treatment with guanidine. Cathepsin II was activated and cathepsin III was completely inactivated or absent from the filtrate. These findings suggest the use of guanidine for the purification of cathepsins I, II, and IV.

DISCUSSION

The experiments presented in this report show that the intracellular kidney cathepsins are inhibited by several carbonyl group reagents. Cathepsin III was completely inhibited by phenylhydrazine in a concentration of 4 millimole/l. and by hydroxylamine in a concentration of 20 millimole/l. Hydrazine was less effective and 200 millimole/l. were required to produce 61% inhibition. Cathepsin II was next in response to phenylhydrazine (89% inhibition by 20 millimole/l.), but the order of effectiveness of hydrazine and hydroxylamine as inhibitors was reversed as compared to cathepsin III. Cathepsins I and IV were inhibited by phenylhydrazine, though to a smaller extent than cathepsins II and III, but showed undiminished activity in presence of hydroxylamine (26 millimole/l.). The four cathepsins were not inhibited by dimedon (6 millimole/l.) or by semicarbazide (12 millimole/l.).

The possibility must be considered that the hydrazines might react with the acid amides, used as substrates for cathepsins II and III, with formation of acid hydrazides through displacement of ammonia. Such a reaction would simulate an inhibition through a decrease in the concentration of available substrate. *This mode of action can be ruled out by the fact that phenylhydrazine inhibited cathepsin III completely and cathepsin II 47% when only 4 moles of inhibitor were added to 50 moles of substrate* (see Table II). The correction for a decrease in the substrate concentration from 50 to 46 millimole/l., assuming quantitative acid hydrazide formation, would change the inhibition of cathepsin II in this example from 47 to 42% and would, therefore, explain only a small fraction of the inhibition observed. It would not alter the 100% inhibition of cathepsin III.

Additional proof that the inhibitors react with the cathepsins, and not with the substrates, was obtained from dialysis experiments, which showed that a considerable degree of inhibition persisted after enzyme-inhibitor mixtures had been dialyzed against 100 vol. NaCl solution for 24 hr.

The groups (or group) in the enzyme molecule which react with the carbonyl group reagents is unknown. The fact that the addition of increasing quantities of cysteine decreased progressively the inhibitory effect of a given phenylhydrazine concentration (Table IV), suggests that the inhibitors and cysteine might compete for the same group of the enzyme molecule. Nothing is known, however, about the nature of the group which reacts with cysteine when it activates these enzymes.

SUMMARY

1. A method is described for the partial purification of hog kidney cathepsins. Fresh kidneys were found more suitable than kidney dry powders for preparing solutions containing all four cathepsins.
2. Phenylhydrazine was found to be a strong inhibitor for kidney cathepsins. In a concentration of 4 millimole/l. it inhibited cathepsin III completely, cathepsin II 47%, cathepsin I 19%, and cathepsin I + IV 17%. Hydrazine was considerably less effective and about 50–100 times higher concentrations were required to produce comparable inhibitions.
3. Hydroxylamine (20 millimole/l.) inhibited cathepsin III completely and cathepsin II slightly (7%). Hydroxylamine (26 millimole/l.) did not interfere with the activities of cathepsins I and IV.
4. Dimedon (6 millimole/l.) and semicarbazide (12 millimole/l.) did not inhibit any of the four kidney cathepsins. Guanidine (1 mole/l.) produced a precipitate in the cathepsin solutions and the resulting filtrate had no cathepsin III activity. Guanidine did not interfere with the activities of cathepsin I, II, and IV.
5. The degree of inhibition caused by a given concentration of phenylhydrazine decreased with increasing enzyme and activator (cysteine) concentration.
6. Dialysis for 24 hr. against 100 vol. 1% NaCl solution caused only slight reactivation of cathepsin preparations which had been incubated with phenylhydrazine.

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The Amino Acid Composition of Tetanal Toxin[1]

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INTRODUCTION

Crystalline tetanal toxin (2), prepared from the filtrates of *Clostridium tetani*, has been shown to be a homogeneous protein (3) containing 15.7% nitrogen, 0.065% phosphorus and 1.04% sulfur. The percentages of 13 amino acids in this protein have been determined in the present studies.

EXPERIMENTAL

Samples of the previously described toxin (3) were hydrolyzed as follows: *Acid Hydrolysis.* A mixture containing 159.1 mg. of (dried) toxin and 5 ml. of 8 *N* HCl was refluxed for 20 hr.

Alkaline Hydrolysis. A mixture containing 39.75 mg. of (dried) toxin and 1 ml. of 5 *N* NaOH was autoclaved for 10 hr. in a sealed tube. The microbiological methods and techniques were those described by Dunn *et al.* (4).

The relatively high reliability of the microbiological assay data is indicated by the mean deviation from the mean values at the different levels of samples which, in the first assay, averaged 2.2% (range, 0.5% for glutamic acid to 7.7% for methionine) and, in the second assay, averaged 2.8% (range, 0.7% for isoleucine to 4.8% for lysine).

Relatively high precision was attained in the two assays (Table I) since the differences between the values found for each amino acid and calculated as per cent of the values found in the first assay, averaged 2.8% (range, 0% for aspartic acid and lysine to 6% for glutamic acid).

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[1] Paper 55. For Paper 54 see Rockland and Dunn (1). This work was aided by grants from the Nutrition Foundation and the National Institutes of Health (U. S. Public Health Service) to one of us (M. S. D.). The authors are indebted to Miss Ruth Malin and Mr. Samuel Eiduson for assistance in the microbiological assays.

TABLE I

Tetanal Toxin: Per Cent of Amino Acids, Minimum Molecular Weight and Minimum Number of Amino Acid Residues per Mole

Amino acid	Amino acid in toxin	Minimum mol. wt. based on one amino acid residue per mole[a] (A)	Minimum amino acid residues per mole[b] (B)	Minimum mol. wt. based on *n* amino acid residues per mole[c]	Amino acid in toxin (corrected per cent)[d]
	per cent				
Arginine	3.38 (3.43,3.33)	5,154	13	67,002	3.36
Aspartic acid	15.1 (15.1,15.1)	882	76	67,488	15.3
Glutamic acid	10.3 (10.0,10.6)	1,428	47	67,116	10.3
Glycine	3.33 (3.30,3.35)	2,255	30	67,650	3.34
Histidine	1.15 (1.16,1.13)	13,495	5	67,475	1.15
Isoleucine	9.3 (9.3,9.2)	1,411	48	67,728	9.36
Leucine	8.3 (8.5,8.1)	1,581	43	67,983	8.23
Lysine	10.0 (10.0,10.0)	1,462	46	67,252	10.0
Methionine	1.79 (1.75,1.83)	8,335	8	66,680	1.78
Phenylalanine	5.0 (5.03,4.95)	3,304	20	66,080	4.91
Threonine	5.1 (5.00,5.19)	2,335	29	67,715	5.13
Tryptophan[e]	0.82	25,902	3	74,706[f]	0.91
Valine	5.4 (5.29,5.50)	2,169	31	67,239	5.39
Average				67,286	

[a] (Molecular weight of amino acid/per cent of amino acid in toxin) × 100.

[b] Calculated on basis of histidine as 5.

[c] (A) × (B).

[d] Molecular weight of amino acid × 100 *n*/67,286 where *n* is the minimum number of amino acid residues given in (B).

[e] Single determination in alkaline hydrolyzate.

[f] Omitted in calculating average.

RESULTS

It is noteworthy that tetanal toxin contains exceptionally high percentages of aspartic acid and lysine compared to other simple proteins. This may account in part for the high surface activity of tetanal toxin, such as its marked ability to adsorb to glass and other surfaces (5). It is also of interest that the aspartic acid content is about 50% greater than that of glutamic acid, and that the percentage of isoleucine exceeds that of leucine (Table I). These relationships are the same in botulinum Type A toxin (6) but are reversed in most other proteins and proteinaceous materials of known composition.

It appears that tetanal toxin has the same qualitative, but different quantitative composition of amino acids as diphtherial toxin (7) and botulinum Type A toxin (6). Since crystalline tetanal toxin has a sedimentation contant of 4.5 S (3), its molecular weight probably is in the range 66,000–74,000, according to the data given by Cohn and Edsall (8). It has been calculated (Table I), on the basis of this premise, that tetanal toxin has a molecular weight of about 67,300 and contains from 3 tryptophan residues to 76 aspartic acid residues per mole.

SUMMARY

The percentages of 13 amino acids in an electrophoretically-pure preparation of crystalline tetanal toxin which exhibited constant solubility have been determined by microbiological methods. High percentages of aspartic acid, isoleucine and lysine were found compared to other simple proteins. Although most proteins contain glutamic acid in excess of the aspartic acid and leucine in excess of the isoleucine, the reverse relationship was found for tetanal toxin. The molecular weight of tetanal toxin was estimated to be 67,286. On this basis, the number of amino acid residues per mole ranged from 3 for tryptophan to 47 for glutamic acid, 48 for isoleucine and 76 for aspartic acid.

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Electrophoretic Analysis of Lamb and Sheep Plasmas and Sera

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INTRODUCTION

Recent electrophoretic studies have shown that there is rather great variation in the distribution of the components of plasma and serum of different species (1–6). In all of these studies it has been found that the buffer influences considerably the electrophoretic resolution of the plasma and serum into their respective protein components. The influence of ionic strength on the electrophoretic analysis of bovine plasma has been demonstrated (9).

The purpose of this investigation has been to study the resolution and relation of the components of lamb and sheep plasmas and sera in phosphate and veronal buffers.

EXPERIMENTAL

The experimental technic used in determining the mobilities and percentage composition of all the plasmas and sera is the same as that described in a previous communication (7).

The phosphate, veronal-NaCl, veronal, and veronal-citrate buffers used in the investigation on lamb and sheep plasmas and sera have been described (7). The conductivities for the phosphate, veronal, veronal-citrate, and veronal-NaCl buffers were approximately 6.75, 3.02, 2.50, and 8.10 × 10^3 mhos, respectively. The time for a run in phosphate buffer was 240 min., in veronal buffer, 150 min., in veronal-citrate buffer, 120 min., and in veronal-NaCl buffer, 285 min. Usually the experiment was continued until the fastest component, albumin, had migrated approximately 6 cm. from the initial boundary. In phosphate buffer a voltage gradient of about 5.85 v./cm. was maintained, in veronal about 8.75 v/cm., in veronal-citrate about 10.55 v./cm., and in veronal-NaCl about 4.90 v./cm.

Both fresh and dried lamb and sheep plasmas were used in this investigation; also both fresh and dried sera of lamb and sheep were used. Approximately 2 l. of blood were collected from 10 spring lambs on the slaughter floor and allowed to coagulate at 5°C. After the clot had synerized (within 2 days), the serum was separated by centrifugation and dried in the frozen state. Samples of the fresh serum were taken for electrophoresis. Approximately 2 l. of blood from the same 10 lambs were collected in a jar containing 150 ml. 4% sodium citrate. The cells were separated from the plasma by centrifugation. Samples of the fresh plasma were taken for electrophoresis. The remainder of the plasma was dried in the frozen state. At a later time, 2 l. of blood were collected from 10 adult sheep on the slaughter floor and allowed to coagulate at 5°C. After the clot had synerized, the serum was centrifuged off and samples of the fresh serum were taken for electrophoretic analysis. The remainder of the serum was dried in the frozen state. Approximately 2 l. of blood from the same 10 animals were collected in a jar containing 150 ml. 4% sodium citrate. The cells were separated from the plasma by centrifugation. Samples of the fresh plasma were taken for electrophoresis. The remainder of the plasma was dried in the frozen state.

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The samples of plasma and serum were prepared for electrolysis as previously described (8). The final concentration of the protein was 2% as determined refractometrically. Duplicate determinations were made in each buffer.

RESULTS

Some boundary pictures of dried plasma in each buffer are given in Fig. 1.

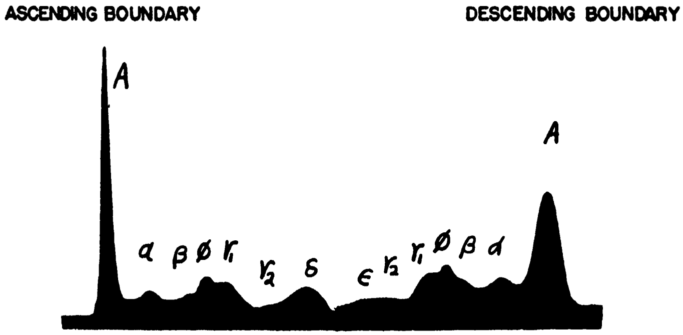
In the phosphate buffer, the ascending and descending boundaries are fair mirror images, although the albumin boundary on the descending side is much broader than that on the ascending side. On the ascending boundary, the components are rather well resolved—at least it is comparatively easy to identify the various components.

The boundaries in veronal-NaCl buffer approach more nearly mirror images, and the resolution of albumin is rather good. The α_1 and α_2 are defined and the β is quite well defined. The γ_1, γ_2, and ϕ are not too well resolved.

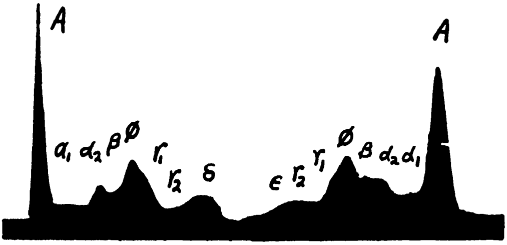
The boundaries in veronal buffer approach even better the ideal mirror image criterion. All components are fairly well resolved with the exception of γ_1 and ϕ.

In the veronal-citrate buffer, fairly good resolution is found; however, there is much to be desired in the resolution of γ_2. The boundary anomalies are rather large.

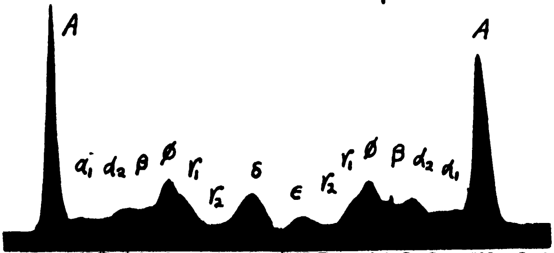
The boundary picture of serum in two buffers is given in Fig. 2. The fibrinogen peak is absent in both buffers indicating that, although it might have superimposed on it some of the γ_1-globulin, it nevertheless migrates for the most part as a distinct entity. Resolution is about the same in both buffers. The descending albumin boundary in phosphate



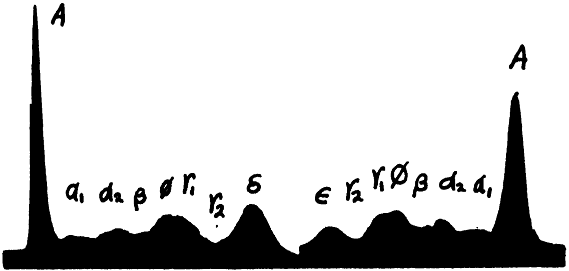
PHOSPHATE BUFFER pH 7.7, Γ/2 0.2



VERONAL-NaCl BUFFER pH 8.5, Γ/2 0.2



VERONAL BUFFER pH 8.6, Γ/2 0.1



VERONAL-CITRATE BUFFER pH 8.5, Γ/2 0.1

Fig. 1. Electrophoretic diagrams of dried sheep plasma.

buffer is broader than the ascending. This seems to be characteristic for phosphate buffer.

In all the buffers, the γ-globulins are well separated from the δ boundary in both serum and plasma. The ϵ boundary seems not to be as distinct in the phosphate and veronal-NaCl buffers, consequently the separation of the γ_2 from this anomaly is more or less arbitrary.

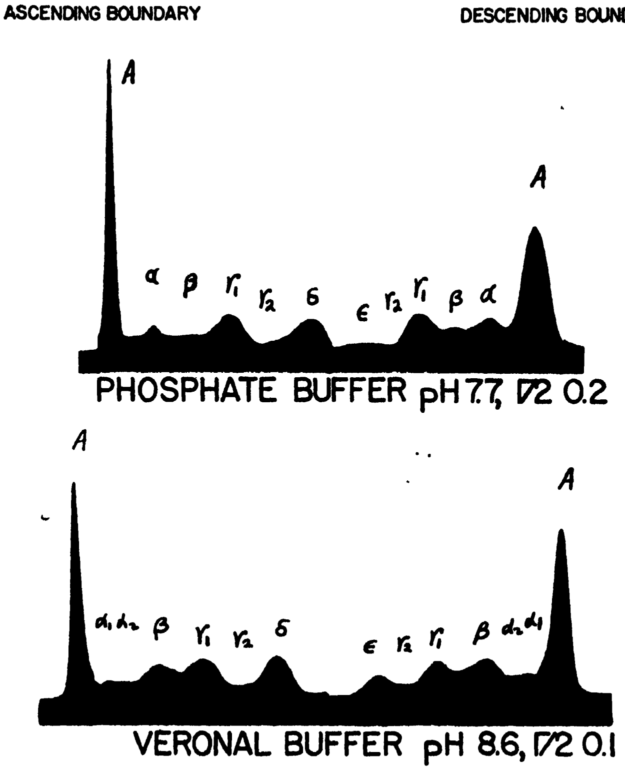


FIG. 2. Electrophoretic diagrams of sheep serum.

Since there was no difference between the mobilities of fresh and dried lamb and sheep plasmas, the values were averaged together for each buffer, and the average values given in Table I. When the number of determinations justified, the standard deviation, σ, was calculated and listed. The same procedure was used in listing the mobilities of fresh and dried lamb and sheep sera. The precision is rather good for all the buffers when it is realized that in the veronal and phosphate buffers, the mobilities for both lamb and sheep fresh and dried plasmas are averaged together. Albumin exhibits the greatest precision. The precision decreases as the components become more difficult to resolve. In phosphate buffer, the γ_2-globulin of the plasma shows the least precision. This is due to the fact that separation is not definite. The serum, on the other hand, shows γ_1 to be less precise. Poor resolution of the γ_1 and γ_2 is responsible for this result.

TABLE I

Mobilities of Plasma and Serum Components

Material	Buffer	pH	$\Gamma/2$	No. of samples	Albumin	α_1	α_2	β	ϕ	γ_1	γ_2
Plasma	Phosphate	7.7	0.2	8	6.14	4.59		3.33	2.72	2.06	0.89
σ					0.10	0.07		0.07	0.06	0.05	0.06
Serum	Phosphate	7.7	0.2	8	6.13	4.61		3.45		2.29	0.92
σ					0.07	0.05		0.07		0.10	0.03
Plasma	Veronal	8.6	0.1	8	6.68	5.32	4.14	3.23	2.54	1.90	1.02
σ					0.03	0.06	0.05	0.03	0.04	0.07	0.09
Serum	Veronal	8.6	0.1	8	6.64	5.28	4.25	3.40		2.27	1.02
σ					0.06	0.07	0.08	0.09		0.15	0.10
Plasma	Veronal-citrate	8.82	0.1	2	6.87	5.42	4.21	3.23	2.49	1.80	0.74
Serum	Veronal-citrate	8.82	0.1	2	7.13	5.71	4.36	3.41		2.32	0.80
Plasma	Veronal-NaCl	8.68	0.2	2	5.48	4.30	3.36	2.71	2.18	1.63	0.75
Serum	Veronal-NaCl	8.68	0.2	2	5.78	4.64	3.67	3.22		2.23	0.96

In the veronal buffer, resolution of the γ_2-globulin of the plasmas and sera is the least precise due to the difficulty in separating this component.

In veronal-citrate and veronal-NaCl buffers, duplicate analyses were made only on the dry sheep serum and plasma.

Inasmuch as there was neither difference between the percentage distribution of fresh and dried plasmas nor between the percentage distribution of fresh and dried sera, the results for each were averaged together and the average values listed for each sheep and lamb in Table II. When there were sufficient determinations the standard deviation, σ, was listed. The average albumin/globulin, A/G, ratios are given, together with the standard deviations. Duplicate determinations were made on each sample in each buffer used. For the most part the results for albumin are about the same, whether analyzed in phosphate or veronal buffers. The values for albumin content of the lamb plasma are significantly greater than those for sheep plasma, whether analyzed in phosphate or veronal buffers. The values for albumin in sheep plasma are somewhat less when analyzed in veronal-NaCl buffer. The values for veronal-citrate buffer are about the same as those for veronal and

TABLE II

Percentage Composition of Plasma and Serum

Material	Buffer	pH	Γ/2	No. of samples	Albumin	α_1	α_2	β	ϕ	γ_1	γ_2	A/G
Plasma (lamb)	Phosphate	7.7	0.2	4	52.6	14.2		6.8	9.7	13.0	3.8	1.11
σ					0.68	0.29		0.16	0.29	0.62	0.63	0.03
Plasma (sheep)	Phosphate	7.7	0.2	4	38.9	16.2		9.5	11.9	17.9	5.7	0.64
σ					1.06	0.88		0.80	0.45	0.27	0.42	0.03
Serum (lamb)	Phosphate	7.7	0.2	4	54.7	14.9		7.2		18 7	4.6	1.20
σ					1.85	1.23		0.69		0.21	0.83	0.09
Serum (sheep)	Phosphate	7.7	0.2	4	40.7	15.8		7.9		31.0	4.6	0.69
σ					0.72	0.79		0.87		0.70	0.26	0.03
Plasma (lamb)	Veronal	8.6	0.1	4	51.6	7.6	9.4	6.7	11.5	11.1	2.2	1.06
σ					0.80	0.41	0.3	0.41	0.47	0.36	0.30	0.03
Plasma (sheep)	Veronal	8.6	0.1	4	38.1	8.2	9.4	10.6	16.9	13.2	3.8	0.61
σ					0.71	0.21	0.41	0.91	0.87	0.90	0.29	0.02
Serum (lamb)	Veronal	8.6	0.1	4	53.6	7.1	10.2	8.0		17.8	3.3	1.16
σ					0.81	1.41	1.31	0.46		0.34	0.12	0.04
Serum (sheep)	Veronal	8.6	0.1	4	39.3	7.6	8.7	8.7		31.7	4.1	0.65
σ					0.67	0.62	0.73	0.50		0.56	0.49	0.02
Plasma (sheep)	Veronal-citrate	8.82	0.1	2	39.4	8.5	10.1	10.3	16.5	12.5	2.9	0.65
Serum (sheep)	Veronal-citrate	8.82	0.1	2	39.6	8.7	11.9	5.8		31.1	3.0	0.66
Plasma (sheep)	Veronal-NaCl	8.68	0.2	2	34.7	9.0	9.4	9.8	17.6	15.0	4.8	0.53
Serum (sheep)	Veronal-NaCl	8.68	0.2	2	37.1	7.9	9.5	6.7		33.5	5.5	0.59

phosphate buffers. The percentage distribution for the rest of the components differs significantly from one buffer to another. In phosphate buffer, the α_1- and α_2-globulins are not resolved; however, in veronal buffer they are.

The percentage distribution of the components of serum shows the lamb serum to possess significantly more albumin than the sheep serum, whether analyzed in phosphate or in veronal buffers. The α-globulin is not resolved into α_1 and α_2 in phosphate buffer but is in veronal buffer. In the sheep serum, the sum of the α_1- and α_2-globulins in veronal buffer is about equal to the α-globulin in phosphate buffer. The sheep serum has significantly more γ_1-globulin than the lamb serum. The rest of the components seem to agree rather well.

DISCUSSION

As Deutsch and Goodloe (3) pointed out, sheep plasma is very difficult to analyze electrophoretically. The analyses reported in Table II are on two separate plasmas and sera; namely, lamb and sheep plasmas and sera. The greatest differences seem to be in the albumin and γ-globulin contents.

Between the phosphate and veronal buffers, there seem to be rather small differences in the values for albumin and β-globulin contents in the same plasma. There is considerable difference in the value for α-globulin content when analyzed in phosphate or veronal buffer. In veronal the α_1- and α_2-globulins are resolved. The amount of fibrinogen is greater in veronal buffer than in phosphate buffer, and there is a correspondingly greater amount of γ_1-globulin in phosphate buffer than in veronal buffer. These differences are probably due to some of the fibrinogen being superimposed on the γ_1-globulin and the difficulty in resolving the fibrinogen and γ_1-globulin in veronal buffer. The low precision in some of the components is not surprising, since some of them had to be arbitratily resolved during the interpretation procedure.

The A/G ratio for the veronal-NaCl buffer is the lowest of all, indicating that the albumin was migrating in a state less contaminated by the other components. The A/G ratios for the phosphate buffer and the veronal-citrate buffer are about the same, whereas the A/G ratio for veronal buffer is slightly less—intermediate between the phosphate and veronal-NaCl buffers.

The percentage distribution of the components of sheep serum are strikingly similar for veronal and phosphate buffers with the exception of the α-globulins. The A/G ratios are about the same. In lamb serum, there is more difference in the percentage distribution of the components for the two buffers. The A/G ratios are about the same, however.

The difficulty in resolving many of these protein components is no doubt due to considerable superimposing of the components on one another. The interaction between the different components is probably rather marked. This interaction probably could be best demonstrated by more variation of the ionic strength than was undertaken in this investigation. No doubt, a considerable part of the γ_1-globulin migrates along with the fibrinogen. Here accurate resolution cannot be expected.

ACKNOWLEDGMENTS

The authors are indebted to the late Professor F. C. Koch for his friendly advice and counsel. To Dr. J. B. Lesh, the authors extend thanks for his criticism of the manuscript.

SUMMARY

Fresh and dry lamb and sheep plasmas have been analyzed electrophoretically in phosphate and veronal buffers. Dry sheep plasma has been analyzed in veronal-citrate and veronal-NaCl buffers. Fresh and dry lamb and sheep sera were analyzed in phosphate and veronal buffers. Dry sheep serum was analyzed in veronal-citrate and veronal-NaCl buffers.

In all the buffers, the albumin was rather well resolved. The veronal-NaCl buffer probably gave the most accurate value.

The fibrinogen seemed to be best resolved in the phosphate and veronal-citrate buffers. In the other buffers, it was inadequately separated from the γ_1-globulin.

The two α-globulins were evident in veronal, veronal-citrate, and veronal-NaCl buffers. The two γ-globulins were evident in all buffers.

There is significantly more albumin in lamb plasma and serum than in sheep plasma and serum for all buffers used.

There is significantly more γ-globulin in sheep plasma and serum than in lamb plasma and serum.

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On the Glyconeogenetic Action of Fed Glycine in the Rat [1]

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INTRODUCTION

In 1938, Mirski *et al.* (1) published the results of their studies on carbohydrate reserves in rats fed either a diet rich in carbohydrate or one high in protein. They found that rats maintained on protein-rich diets exhibited markedly higher glycogen levels following stress than did animals maintained on carbohydrate-rich diets. This they termed the "protein effect." Some time later, investigations were undertaken here to determine the extent to which individual amino acids, fed as part of an otherwise satisfactory diet, were capable of exerting the "protein effect" (2). It was found that under our experimental conditions L-leucine and L-glutamic acid exerted no "protein effect," DL-alanine showed a slight effect, while glycine exerted a marked "protein effect." Animals fed on diets containing 10–15% glycine for two days showed liver glycogen levels of more than 1% after a 24 hr. fast as compared with liver glycogen levels of about 0.3% in animals previously fed the control diet with no added glycine.

Later, a report from this laboratory presented data on the effects on carbohydrate storage of a large dose of insulin in rats prefed on diets with and without added glycine (3). Following an 8 hr. fast, the animals were given 12 units of insulin per kg. of body weight. After another 5 hr. the animals prefed the glycine diet showed 2.5 times as much muscle glycogen and 10 times as much liver glycogen as the control-fed rats. The depression of blood sugar was more marked in the latter animals.

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In view of this great difference in carbohydrate stores following insulin action, it seemed desirable to determine the extent of body storage of glycine in animals fed the two diets, at a time comparable to that at which insulin was administered in the report just referred to, *i. e.*, following an 8 hr. fast. Analyses of free and total glycine in blood, liver, muscle, intestine and kidney are reported herein. The data indicate that insufficient glycine is available, even if all the carbon atoms of this amino acid were to be converted to carbohydrate, to account for the differences in glycogen found (3) following the action of insulin.

EXPERIMENTAL

Rations

The control ration has the following percentage composition: casein, 16; Wesson salt mixture (4), 5; Brewer's yeast (Squibb), 10; cod liver oil, 2; Wesson oil, 5; commercial white corn dextrin, 54; and dextrose, 8.

The experimental ration differed from this only in that 10% of glycine was substituted for an equal weight of dextrin. Thus, a 10% glycine diet refers to one in which there is 10% of glycine and 44% of dextrin with the other ingredients present in the same proportions as in the control diet.

Animals

Male albino rats of the Sprague-Dawley strain (approximately 200–300 g.) were used.

Methods

Glycine was determined by the method of Alexander, Landwehr and Seligman (5).

Preparation of Tissues for Free Glycine Determination. An iron filtrate (6) was used for the determination of free glycine in blood. For the determination of free glycine in the other tissues, a sample of tissue (0.4–1 g.) was minced into a weighed 6 in. test tube. The tube was reweighed and the tissue ground in 2 ml. of water with a modified Potter-Elvehjem homogenizer. After transferring the homogenate to a 50 ml. centrifuge tube and diluting to about 30 ml. with water, 2 ml. of 20% trichloroacetic acid were added to precipitate most of the protein. The precipitate was removed by centrifugation, and then resuspended in about 25 ml. of water containing another ml. of 20% trichloracetic acid. The centrifugation was repeated. These supernatants were combined and diluted to 100 ml. with water. Glycine was determined on 5 ml. aliquots.

Preparation of Tissues for Total Glycine Determination. For total glycine in blood, 0.5 ml. of blood was laked in 11.2 ml. of water and 3.3 ml. of concentrated H_2SO_4 were added to make the final acid concentration about 8 *N*. For the other tissues, a sample varying in size from 0.2 to 0.5 g. was minced and transferred to a weighed flask containing 15 ml. of 8 *N* H_2SO_4. After reweighing the flasks, the tissues were hydrolyzed by boiling under reflux for 18–24 hr. The hydrolyzates were transferred to 200 ml. volumetric flasks and diluted to the mark. An aliquot was transferred to a 50 ml. centrifuge tube containing enough crystalline $Ba(OH)_2$ to neutralize most of the acid, but not enough to render the solution alkaline. The centrifuge tube was then fitted with a stopper bearing a capillary tube and heated for 0.5–1 hr. to complete the reaction and to digest the $BaSO_4$. After cooling, the pH was checked to make sure the solution was still acid, the $BaSO_4$ was centrifuged down, and the glycine determination carried out on a 5 ml. aliquot of the supernatant fluid.

Plasma protein was determined by the method of Weichselbaum (7).

Total nitrogen was determined by the micro Kjeldahl procedure using aliquots of the hydrolyzate prepared for the determination of total glycine.

Tissue water was determined by heating a weighed sample to constant weight at 100–105°C.

Plan of a Typical Experiment

The animals were placed on control ration for 24 hr. prior to the beginning of the experimental period to accustom them to synthetic rations. This procedure leads to improved food consumption during the experimental period. Following this preparatory period, the rats were given the glycine diet or kept on the control diet for 38 hr., at which time the last meal was given. One to two hr. were allowed for the consumption of the last meal and the rats were sacrificed 8 hr. later. Animals on the control diet were pair-fed with animals on the glycine diet. Food consumption was 12–15 g./animal /day.

Sacrifice was accomplished by decapitation. Blood was collected in an oxalated beaker and the other tissues were removed at once.

RESULTS

Table I summarizes the data obtained on the glycine content of the various tissues.

TABLE I

Free and Total Glycine in Tissues of Animals Prefed Control Diet or Glycine Diet Expressed as Wet Weight of Tissue

Tissue	Free glycine Animals prefed		Total glycine Animals prefed	
	Control diet Mg.-%	Glycine diet Mg.-%	Control diet Mg.-%	Glycine diet Mg.-%
Blood	3.7 (6)	8.1 (6)	483 (9)	533 (9)
Kidney	77 (6)	79 (3)	434 (11)	439 (9)
Intestine	76 (6)	103 (6)	396 (10)	444 (11)
Liver	57 (6)	77 (4)	459 (11)	476 (11)
Muscle (Gastrocnemius)	55 (6)	133 (5)	390 (11)	471 (11)

Values are averages for the numbers of animals shown in parantheses.

Blood. The blood of the glycine-fed animals contained a little more than twice as much free glycine as the blood of the control-fed animals. This difference of 4.4 mg.-% is not sufficient, however, to account for the difference of 50 mg.-% noted in the total glycine content of the two types of blood. It is assumed that the excess glycine found in the blood of the glycine-fed animals is present in combined form.

Only insignificant differences in plasma proteins and total protein were found.

Kidney. The differences in free and total glycine found here cannot be considered significant. The protein content of kidney tissue was lower in glycine-fed animals than in the controls (17.2% compared with 19.0% for control-fed). This apparent difference largely disappears, however, when allowance is made for the somewhat higher water content of this tissue in glycine-fed rats (77.6% compared with 76.3% for control-fed).

Intestine. A small excess of total glycine may be noted in the intestine of the glycine-fed animals. Of this excess, nearly 60% can be accounted for as free glycine. The remainder must be assumed to be in combination.

No difference was found in the total protein contents of the intestines of the animals on the two diets.

Liver. A very small difference in total glycine was found in this tissue and the excess may all be accounted for as free glycine.

Insignificant differences in total protein were found for tissues from rats on the two diets.

Muscle (gastrocnemius). A relatively large difference in total glycine was found in muscle and it can all be accounted for as free glycine.

The differences in total protein and water content were not significant.

In some experiments, a 15% glycine ration was fed. The data on tissue glycine were strictly comparable to those found when the 10% glycine diet was fed. Also, carbohydrate storage after a 24 hr. fast is comparable when 10 or 15% glycine diets are employed (2).

DISCUSSION

It is the purpose of this paper to indicate that the results reported earlier (3) on carbohydrate storage following an 8 hr. fast and a 5 hr. period of insulin action, in glycine-fed animals, cannot be explained by a direct conversion of stored glycine to carbohydrate. The calculations show that insufficient glycine is present in the glycine-fed animals (Table I) after an 8 hr. fast to account for the extra carbohydrate present in similarly treated animals following the action of insulin (3). Table II adapted from our earlier paper (3) shows the extent of carbohydrate storage in glycine-fed and control-fed animals. Table III gives the calculations based on our findings reported here and on the data from Table II.

TABLE II

The Effect of Prefeeding Extra Glycine on the Response of Rats to 12 Units of Insulin/Kg. as Measured by Alterations in Blood Sugar, and Muscle and Liver Glycogen

Adapted from Cunningham, Barnes and Todd (3)

	Glycogen (% wet wt.)				Blood sugar Mg.-%	
	Control ration		Glycine ration		Control ration	Glycine ration
	Muscle	Liver	Muscle	Liver		
Before insulin	0.43	3.89	0.63	3.51	114	133
After insulin	0.21	0.13	0.52	1.36	66	106

From a theoretical consideration, it may be deduced that, if glycine were completely converted to carbohydrate, the weight of carbohydrate produced could be no more than about $\frac{60}{75}$ of the weight of glycine available (glycine to glycolaldehyde, followed by condensation—it is not inferred that this is the mechanism involved). If, then, the extra glycine found in the body of a glycine-fed animal after an 8 hr. fast (Table III) were completely converted to carbohydrate during 5 hr. of insulin action, this would account for $\frac{60}{75} \times 85$ or around 68 mgs. of carbohydrate. But, as seen in Table III, the glycine-fed rats have in their bodies, after an 8 hr. fast and 5 hr. of insulin action, an excess of some 407 mg. of carbohydrate over that found in control-fed animals. This is about 6 times the quantity of carbohydrate that could be formed by complete conversion of the extra glycine present.

While we have not examined all the body tissues for glycine, those which we have studied comprise about 60% of the body weight and it seems highly unlikely that analyses of other tissues would uncover a storehouse of this amino acid sufficient to account for the differences noted.

TABLE III

Summary of Calculations Showing (1) Excess Glycine Stored in the Body of a Glycine-Fed Rat After 8 Hours Fast, and (2) the Excess Carbohydrate Found in the Body of a Glycine-Fed Rat After 8 Hours Fast and 5 Hours of Insulin Action

Calculations are for a 200 g. rat

1. Tissue	2. Body weight	3. Tissue wt. in a 200 g. rat	4. Excess total glycine in glycine-fed rat 8 hrs. fast.[a]	5. Column 4 calculated to a 200 g. rat	6. Excess carbohydrate in glycine-fed rat after insulin[b]	7. Column 6 calculated to a 200 g. rat
	per cent	*g.*	*mg.-%*	*mg.*	*mg.-%*	*mg.*
Blood	6.7[c]	13.4	50	6.7	40	5.4
Kidney	0.9[d]	1.8	5	0.1	—	—
Intestine	4.0[d]	8.0	48	3.8	—	—
Liver	5.0[d]	10.0	17	1.7	1230	123
Muscle	45.0[d]	90.0	81	72.9	310	279
Totals				85.2		407.4

[a] From Table I.

[b] From Table II.

[c] From Griffith (8).

[d] Approximate values calculated from the data of Donaldson (9).

The diets and feeding procedures were similar in the work reported here, and in that previously reported (3), with one exception: in the earlier work, part of the last meal was given by stomach tube, whereas for the present experiments all of the last meal was fed in the usual manner. This slight variation in technique is believed to have no bearing on the results or calculations.

The calculations indicate that mechanisms other than direct conversion to carbohydrate are involved in the "protein effect" due to feeding extra glycine. As pointed out previously (3), reduced glycogenolysis in the glycine-fed rats cannot be considered a likely factor in accounting for the increased carbohydrate reserves. We also reported (2) that adrenalectomy abolishes the "protein effect" of glycine in rats.

It would appear that glycine in some way stimulates glyconeogenesis, perhaps through increased adrenal cortical activity.

SUMMARY

The free glycine, total glycine, and total protein contents of blood, kidney, intestine, liver, and muscle of animals fed on the glycine diet and on the control diet have been determined. Plasma proteins of blood and the water content of muscle and kidney from animals on each diet were also determined.

In kidney, liver, and muscle, the excess total glycine found in the glycine-fed animals can be accounted for as free glycine. In blood and intestine, some of the excess total glycine found in the glycine-fed animals is apparently present in combined form.

No significant differences in total protein were found, except in kidney, where the decreased total protein of the glycine-fed animals is probably due to the slightly increased water content of the kidneys of glycine-fed animals. No difference in plasma protein was found.

Calculations are outlined which indicate that animals prefed the glycine diet have in their bodies, after an 8 hr. fast and 5 hr. insulin action, about 6 times as much carbohydrate as can be accounted for by direct conversion to carbohydrate of the extra glycine present before the action of insulin.

The results are interpreted as further evidence in support of the theory of stimulated glyconeogenesis as a result of glycine feeding, possibly through increased adrenal cortical activity.

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Electron Microscopic and Electrophoretic Study of Two Strains of Tobacco Mosaic Virus

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INTRODUCTION

Recently (1) a strain of tobacco mosaic virus (*Marmor tabaci* Holmes), designated 1C, was obtained, which regularly induces the formation of intranuclear inclusions in various hosts (tobacco, tomato, pepper). Unlike previously reported plant virus intranuclear inclusions (2,3,4), those induced by this tobacco mosaic strain are very unstable, going readily into solution when the cell is damaged. The nuclear inclusions appear to be of a proteinaceous nature, and behave in general like the cytoplasmic inclusions which are also present. Because of the apparently unique nuclear inclusion-forming property of this new strain (1C) of tobacco mosaic virus, a study of some of its physical properties was undertaken. For purposes of comparison, a typical green mosaic strain of tobacco mosaic virus was employed. This strain was was designated 2A. These physical properties are the size and shape obtained from the electron micrographs of both aggregated and unaggregated material and the electrophoretic mobility of 1C compared to 2A. A biological assay was run on electrophoretically fractionated samples.

Electron microscopic studies on the size and shape of tobacco mosaic virus have indicated a most probable size of 15 mμ × 280 mμ (5,6,7). Sedimentation-viscosity, sedimentation-diffusion and viscosity-diffusion measurements have yielded 13.6, 13.8, and 14.0 mμ, respectively, for the diameter of the rod-shaped particle (8). Since the present estimations of size were made with the electron microscope, the above value of size as obtained with this instrument has been used as a basis for comparison. Aggregation end to end (9), as well as side to side (7), may be produced by salt precipitaiton at room temperatures and strong acid, respectively.

According to Eriksson-Quensel and Svedberg (10), the isoelectric point of common tobacco mosaic virus is 3.49, the relation between electrophoretic mobility and pH in 0.02 *M* acetate being linear. Loring and Stanley (11) obtained a value for the isoelectric point of 3.2–3.5, depending on buffer and strength.

METHODS

The viruses were isolated by a modification of the procedures previously employed (12,13). Following extraction of the fresh tissue with approximately 0.01 *M* phosphate buffer at pH 7.0 plus 0.5% thiourea, the pH of the filtered extract was reduced to 4.3 to denature the chromoprotein complex. The pH was then raised to 7.0 and the extract filtered through Celite. Subsequent precipitations with half-saturated $(NH_4)_2SO_4$ and elutions from Celite were carried out essentially as in previous procedures. The final step in the procedure was dialysis against distilled water. The temperature throughout was held at 0–5°C.

For the estimation of particle length, the usual methods utilizing the R.C.A. universal electron microscope with metal shadowing by a high vacuum evaporator was used. Negatives were projected on a screen for particle measurement. Lengths were measured on a sufficient number of particles to obtain a ferquency distribution. All measurements were made near the central part of the field of view to avoid lens distortion errors.

In estimating the diameter of the particles, the method used (14) was to select portions of the specimen screen in which several virus rods were lined up side by side, dividing the total width by the number of particles in the group. Methods were also worked out for the measurement of diameters on single particles, after metallic shadowing, which will be described elsewhere.

For eletrophoresis, the virus preparations were dialyzed against a suitable buffer for 2 days. The material was then put in the standard double length Tiselius cell and voltage applied for 3 hr. The temperature was 3.2°C.

RESULTS

Electron microscopic examination of 2A, on material in distilled water which had stood in the refrigerator with toluene as preservative for 6 months, gave no significant difference from the same preparation in the freshly prepared state. The predominant number of particles were, as usual, approximately 15 × 280 mμ, Figs. 1,2.

The figures selected for publication in this paper were primarily for illustrating the length characteristics of the virus particles. Measurements of diameters were made on photographs not here shown.

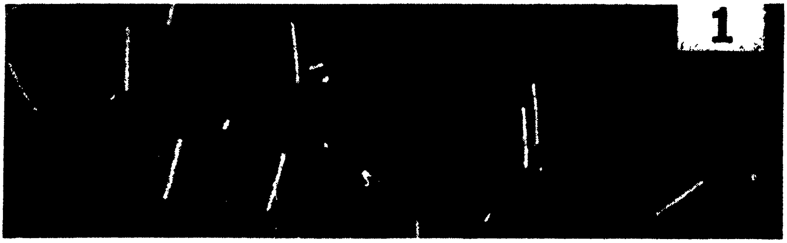

FIG. 1. A dilute 2A preparation in distilled water, virus from tissue infected 7 days.

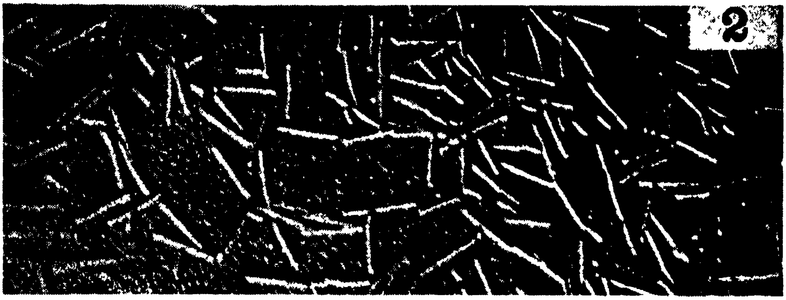

FIG. 2. Same preparation, higher concentration in distilled water.

FIG. 3. Dilute 1C in distilled water.

Similar photographs made on 1C (nuclear strain) (Fig. 3) again showed the usual size particles, a result analogous to that previously found for other strains of tobacco mosaic virus (15,16,17).

These materials were then allowed to stand for 2 hr. in 5% $(NH_4)_2SO_4$ at room temperature, after which the solutions were dialyzed against distilled water for 1 day with frequent changes of water. Photographs were then taken of both preparations (Figs. 4,5). These photographs indicate that many of the virus particles have aggregated end to end giving lengths up to 4 or 5 times the original. Here again there was no difference noted between the 2 strains in this aggregation phenomenon.

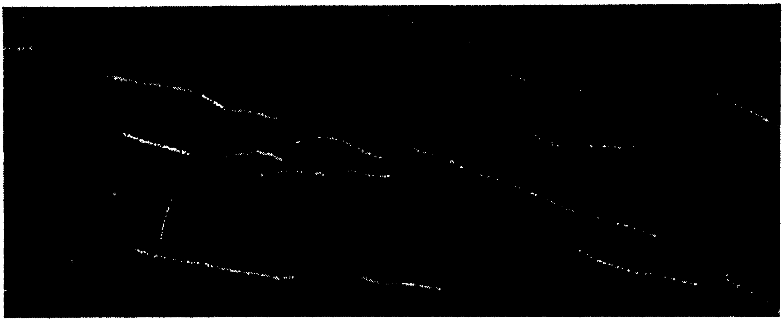

FIG. 4. Same as Fig. 1 except 2A virus kept in 5% $(NH_4)_2SO_4$ for 2 hr. Strong end to end aggregation.

FIG. 5. Dilute 1C treated same as material shown in Fig. 4.

Similar, though less extreme, results were obtained when each virus was allowed to stand for 2.5 hr. in phosphate buffer $M/15$ pH 6.8 to which was added 4% KCl, after which it was dialyzed against distilled water for 18 hr.

The frequency distribution graph for unaggregated 2A, 7-day infection, is illustrated in Fig. 6, 460 particles being included in the measurements. The class interval was taken as 17.2 mμ (a figure originally ar-

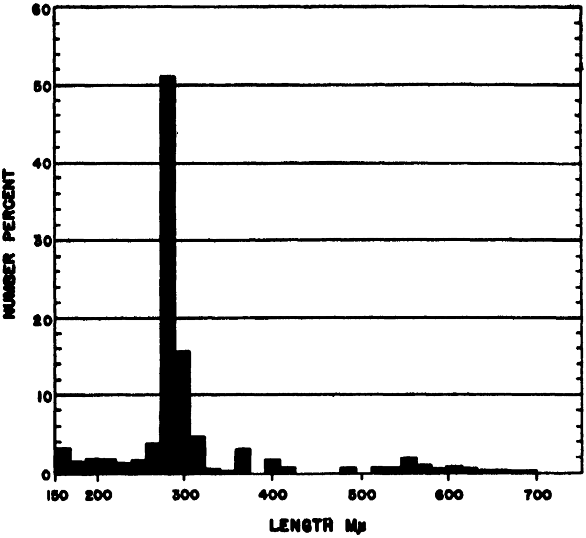

FIG. 6. Frequency distribution of 7 day infection 2A virus chemically purified. Class interval 17.2 mμ, number of particles included 460.

rived at by Rawlins, Robert and Utech (18) as the total error of measurement) in order to compare the distribution of lengths onma terial purified chemically with that purified by the ultracentrifuge (16,17). This graph, when compared with the cited references, indicates that materials prepared by 2 distinctly different methods give somewhat similar frequency distributions of length.

If the ratio of number of particles in the interval 271.4–288.6 mμ to the total number longer than 271.4 mμ is taken as an index of aggregation, the results tabulated in Table I are obtained. From this table it

TABLE I

Number Distribution in Two Frequency Group Intervals of Strains 2A and 1C in the Unaggregated (1st two line) and Aggregated States (lines 3–6)

Material	Number per cent $100 \times \frac{271.4 \text{ to } 288.6 \text{ m}\mu}{271.4 \text{ to } \infty}$	Number per cent $100 \times \frac{288.6 \text{ to } \infty}{271.4 \text{ to } \infty}$
2A	67	33
1C	71	29
2A (Phosphate KCl)	27	73
1C (Phosphate KCl)	25	75
2A (Sulfate)	12	88
1C (Sulfate)	19	81

is evident that, in the presence of strong salt, the number of particles longer than 288.6 mμ is increased at the expense of the shorter particles, and that no significant difference between the two strains exists.

As a test of the steps in the preparative technic of these materials the the effect of temperature on aggregation velocity was also investigated. The starting material was the fresh juice expressed from leaves, infected with 2A virus, which had been frozen 20 hr. This juice was filtered and cleared in a centrifuge at 20,000 × *g* for 5 min. It was then diluted 20 times with 0.01 *M* phosphate buffer of pH 6.86. Three equal fractions of this material were then adjusted so that they contained, respectively, 0% KCl, 0.5% KCl, and 2.5% KCl and then put in the refrigerator at 4°C. Three more similar fractions were kept at room temperature (26°C). After standing 2 days under those conditions, electron microscopic photographs and length measurements were made on all 6 fractions. The results indicated that the aggregation velocity increased with salt strengths and increasing temperatures.

From the results of Lauffer (8), who obtained a linear relationship between the reciprocal of sedimentation velocity and concentration of virus (up to moderate concentrations), it may be concluded (20) that concentration of virus does not change the frequency distribution of particle lengths.

The technic of precipitation with $(NH_4)_2SO_4$ at low temperatures would appear to be successful because of the low temperature and short time in which the virus is exposed to the high salt concentration.

Solutions of 2A were made up in 0.1 *M* sodium acetate buffer at pH 4.92 and 4.20. From mobilities determined in these solutions an isoelectric point of 3.5 was obtained, agreeing with that of Quensel and Svedberg (10).

In phosphate buffer at pH 6.86 (ionic strength 0.1) it was found that the mobility of 1C was only 75% of that shown by 2A in separate runs in which the concentration of 1C was only 1/10 that of 2A. Knight and Lauffer (19) showed that rib-grass strain of tobacco mosaic virus had 87% of the mobility of the common strain in 0.05 *M* phosphate bufler at pH 7.1. To check this behavior biologically, samples of 2A and 1C in phosphate at pH 6.86 were pooled and then after a 3 hr. run in the electrophoresis cell the faster moving 2A fraction pipetted off from the top of the ascending column and the slow moving 1C taken from the top of the descending column. These samples were then subject to bioassay in tobacco plants.

For biological assay the original sample of virus 2A contained approximately 1.25 mg. of protein/cc. and the 1C sample approximately 0.10 mg. of protein (virus plus impurities)/cc. One cc. of the pooled sample was added to 9 cc. of 0.005 *M* phosphate buffer (pH 7.0), carborundum added, and 10 leaves of *Nicotiana tabacum* var. Maryland medium broadleaf inoculated by rubbing.

Seven days after inoculation primary lesions caused by both viruses were visible. Starch-iodine tests were run to distinguish between 1C and 2A infections (Fig. 7). There was a total of 98 1C lesions and 970 2A lesions on the 10 leaves inoculated. This was to be expected, since the

Fig. 7. Leaf of Maryland medium broadleaf tobacco infected with tobacco mosaic viruses 1C and 2A. Leaf decolorized with alcohol and stained with iodine-potassium iodide solution. Note light colored starch-free areas in 1C lesions and excess starch (darkest areas) in 2A lesions.

protein contents of the original virus samples indicated a considerable excess of virus 2A.

Each of the samples obtained from the electrophoresis cell was diluted with 9 parts of pH 7.0 phosphate buffer, carborundum added, and half-leaf comparison inoculations made to Maryland medium broadleaf tobacco. Starch-iodine determinations of primary lesions were run as in the case of the combined preparations. The results are presented in Table II. It is obvious that electrophoresis resulted in a considerable separation of the two viruses.

TABLE II

Biological Analysis of Samples of Tobacco Mosaic Virus Obtained by Electrophoresis of a Mixture of Strains 1C and 2A

Plant number	Slower moving electrophoretic fraction (1C)		Faster moving electrophoretic fraction (2A)	
	1C lesions	2A lesions	1C lesions	2A lesions
1	65	19	1	149
2	60	10	6	238
3	54	6	0	102
4[a]	69	11	5	231
Totals	248	46	12	720

[a] Samples from 1C and 2A fractions run on different plants.

ACKNOWLEDGMENTS

Acknowledgment is made to Mr. R. V. Eck for preparation of some of the virus material and a modification of the isolation procedure; to Mr. B. J. Lloyd, Jr., for taking some of the electron micrographs, and to Mrs. J. L. Bates for electrophoretic data.

SUMMARY AND CONCLUSIONS

Two strains of tobacco mosaic virus, 2A and 1C, were examined under the electron microscope and found to be similar to the normal in length (15 × 280 mμ). The frequency distribution in lengths for material prepared by a chemical method did not differ appreciably from previously published distributions obtained with virus purified by centrifugation. Both strains of virus became aggregated end-end when exposed to 5% $(NH_4)_2SO_4$ for 2 hr. at room temperature, followed by

dialysis against distilled H_2O. Similar results were found using phosphate pH 6.8 to which was added 4% KCl. The aggregation of tobacco mosaic virus 2A was found to be more rapid at 26°C. than at 4°C. Thus, no significant difference was seen in size or shape under different conditions. However, in the Tiselius electrophoretic cell 2A was found to have a higher mobility than 1C. A pooled sample was fractionated by electrophoresis and the two components were found, by testing in plants, to yield their characteristic lesions.

The similarity in size and linear aggregation velocity between the two strains, on the one hand, suggests similar structures at the ends of the particles; on the other hand, differences in electrophoretic mobility indicate a difference in structure affecting total surface charge.

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Selenate Inhibition Studies. III. The Role of Sulfate in Selenate Toxicity in Yeast[1]

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INTRODUCTION

In a previous communication (1) it was reported that selenate toxicity in yeast could be partially reversed by methionine. This seemed to bear a superficial resemblance to reversal of sulfonamides by methionine (2). Moreover, the fact that methionine must be synthesized from sulfate in media which utilize this anion as the sole sulfur source, in addition to the present belief that *p*-aminobenzoic acid (PABA) is concerned in methionine synthesis (2, 3), suggested a relationship between the action of selenate toxicity, sulfonamide inhibition and methionine synthesis.

EXPERIMENTAL

The organism used was *Saccharomyces cerevisiae* (Fleischmann). It was cultured as described (1). The basal medium employed was similar to that used in previous studies (1), except that the sulfate was eliminated as completely as possible. Separate additions of sulfate were made, as desired for each experiment, in the form of $Na_2SO_4 \cdot 10\ H_2O$.

RESULTS AND DISCUSSION

The concentration of sulfate necessary to restore growth to half-maximum (obtained from Fig. 1) in the presence of varying concentrations of selenate is shown in Table I. The data indicate that a relatively

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[2] Swift and Company Fellow in Biochemistry.

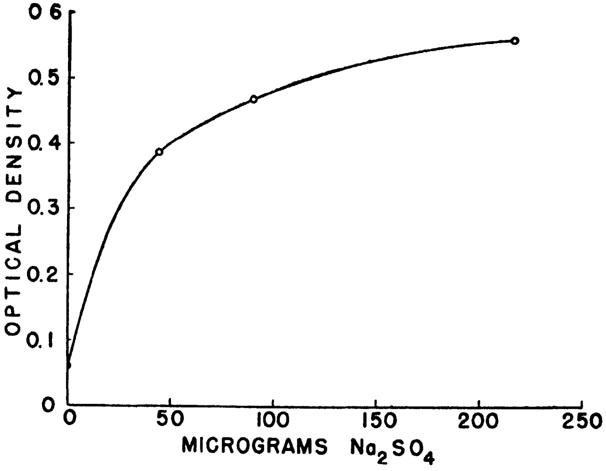

FIG. 1. Growth response of *Sacc. cerevisiae* to added sulfate.

TABLE I

Molar Ratios of Selenate-Sulfate Concentrations at Half Maximum Growth of Sacc. cerevisiae (FB Strain)

H_2SeO_4 *mg.*	Na_2SO_4[a] *mg.*	*I/S* (Molar)
0.50	0.26	1.9
0.50	0.37	1.3
1.00	1.14	0.93
1.00	1.17	0.88
1.00	1.54	0.67
1.50	0.93	1.60
1.50	1.70	0.90
1.50	1.66	0.92
2.00	1.37	1.40
2.00	1.37	1.40
4.00	2.28	1.70
4.00	3.27	1.20
		Av. = 1.1

Incubation time—24 hr.

[a] Sulfate concentrations are stated in terms of the anhydrous salt.

constant ratio exists between the inhibitor selenate (I) and the substrate sulfate (S) necessary to counteract it. This ratio (about unity for half-maximum growth) is direct evidence (4) for the existence of such a competition between selenate and sulfate for an enzyme surface, and it favors the placement of these two substances in direct opposition to one another in the synthesis of methionine. It may be inferred from this, and from the ability of methionine to reverse selenate inhibition (1), that the mode of action of selenate toxicity in yeast is a blocking of methionine synthesis. This confirms and extends observations in plants (5, 6), in which sulfate was found to counteract selenate toxicity.

Evidence that selenate and sulfanilamide operate at different points in preventing methionine synthesis was obtained from the observation that sulfate ($Na_2SO_4 \cdot 10\ H_2O$) in quantities up to 10 mg. per tube had no influence upon the growth inhibition caused by 1–3 mg. of sulfanilamide, whereas 0.1 mg. of *p*-aminobenzoic acid was sufficient to completely overcome this inhibition. On the other hand, *p*-aminobenzoic acid, in amounts up to 2 mg. per tube, was without effect upon any inhibitory concentrations of selenate.

Since the synthesis of methionine requires contributions from both carbon and sulfur sources, it appears from the above observations that the action of selenate under the present experimental conditions is confined to the sulfur source. Since animals do not utilize sulfate appreciably for synthetic purposes (7), *p*-aminobenzoic acid presumably acts on the carbon precursors, and the action of sulfonamides is probably localized there.

2-Chloro-4-aminobenzoic acid, which is considered to be a specific inhibitor of methionine synthesis in *E. coli* (3, 8, 9) was found to be without effect on yeast, even in concentrations up to 4 mg. per tube. It would appear from this observation that the routes of methionine synthesis are different in the two organisms. These differences are emphasized further by the contrasting ability of methionine (yeast (1)) and cystine (*E. coli*) to reverse selenate; methionine is ineffective in the latter organism,[3] and therefore it would seem that the suggestion that methionine is synthesized from cystine in *E. coli* (10) does not hold for yeast. Additional evidence in this connection is that methionine and not cystine can be used as a satisfactory source of sulfur in yeast.[4] Schultz and Pomper (11) have recently shown that, of 40 species of *Saccharomyces* (including *Sacc. cerevisae*), none could utilize cystine for growth, whereas (in contrast to *E. coli* (10)) 36 species could utilize methionine.

[3] *Arch. Biochem.* in press.

[4] Unpublished data.

It should be emphasized that the action of selenate in blocking the conversion of sulfate to methionine is operative only when sulfate is the sole source of sulfur. From the experiments with methionine (1) it is believed that processes other than the synthesis of this amino acid are concerned, since complete reversal of selenate toxicity is never realized with methionine.

ACKNOWLEDGMENT

The authors are grateful to Dr. J. B. Strandskov for a sample of 2-chloro-4-aminobenzoic acid.

SUMMARY

1. A relatively constant ratio has been found for a given concentration of selenate causing inhibition, and for the amount of sulfate necessary to reverse it. This suggests that the mode of action of selenate toxicity in yeast is a competitive inhibition between sulfate and selenate resulting in the blocking of methionine synthesis.

2. Sulfate and *p*-aminobenzoic acid are without influence on sulfonamide and selenate inhibition, respectively. This indicates that these inhibitors operate at different loci in the inhibition of methionine synthesis.

3. No inhibition has been observed in yeast with 2-chloro-4-aminobenzoic acid, contrary to results previously obtained with *E. coli*.

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Studies on the Ultraviolet Absorption Spectra of Collagen

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INTRODUCTION

The possibility of characterizing the purity of collagen preparations from their absorption in the ultraviolet is suggested by the fact that amino acid analyses of collagen (1) indicate an essentially complete absence of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, which are responsible for the characteristic absorption of proteins in the 250–290 mμ range of the ultraviolet (2,3,4). The fact that purified gelatin (5) obtained on partial hydrolysis of collagen shows none of the absorption in the region from 250–290 mμ characteristic of these aromatic amino acids lends further credence to the thesis that purified collagen might, like gelatin, lack characteristic absorption bands in this region of the ultraviolet spectrum.

METHODS AND RESULTS

The absorption in the ultraviolet of the protein preparations was measured with a Hilger quartz spectrograph (Model E 484) equipped with a Spekker photometer using a tungsten spark source. The photographic plates were projected and the match points at different wavelengths determined visually.

The first studies were made on rat-tail collagen in view of its initial relatively high state of purity and because it could be dissolved in dilute acetic acid. The collagen was purified according to Bergmann and Stein (6) by successive prolonged extraction with Na_2HPO_4 in the cold room, followed by ether extraction. The material was then dissolved in dilute acetic acid and reprecipitated with NH_4OH. The precipitate was twice extracted for 12 hr. with Na_2HPO_4, washed with water and redissolved in 0.05–0.1% acetic acid. The collagen was then precipitated by adding NaCl solution to make the final salt concentration 0.75–1%. The precipitated material was washed with water containing a trace of ammonia and then dissolved in 0.1% acetic acid (final pH 3.32) at a concentration of 0.076%. The absorption in the ultraviolet is shown in Fig. 1 (Curve 1), from which it appears that the absorption curve of this preparation re-

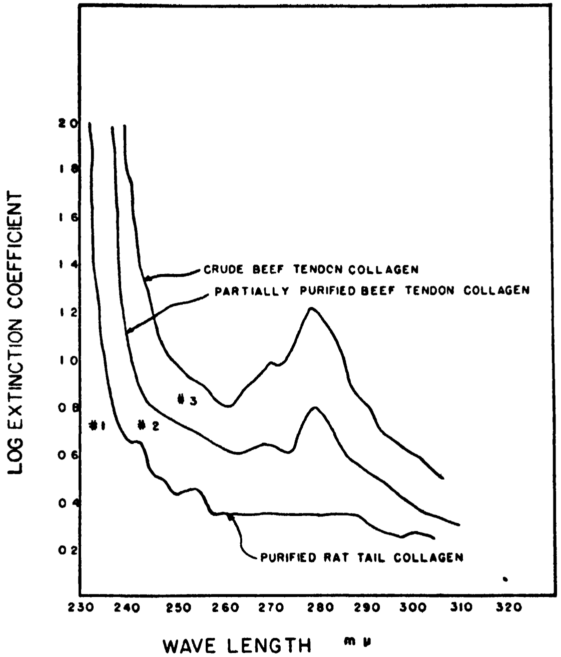

FIG. 1. Ultraviolet absorption spectra of collagen in different stages of purification. Curve 1. Rat-tail collagen highly purified according to a modification of Bergmann and Stein (6). Curve 2. Beef tendon collagen after successive prolonged extractions with Na_2HPO_4. Curve 3. Beef tendon collagen after a single extraction with Na_2HPO_4.

sembles closely that characteristic of purified gelatin (5). The complete lack of absorption peaks from 257.5 to 287.5 mμ can be interpreted as indicating the absence of phenylalanine (maximum absorption at pH 4.3 at 258 mμ), tryptophan (maximum absorption at pH 4.3 at 278 mμ), and of tyrosine (maximum absolption at pH 4.3 at 275 mμ), or of proteins which contain any of these aromatic amino acids The rise in absorption below 240 mμ is that characteristic of the non-aromatic amino acids in collagen. The small irregularities in the curve from 240 to 257 mμ are probably due to impurities, since the absorption in this region varies somewhat with different preparations of rat-tail collagen. Although the nature of the contamination is unknown, it might be suggested that certain purines or pyrimidines (*e.g.*, guanine at pH 6.5 has one of its maxima at 246 mμ, see 3) could account for some of these irregularities in the curve.

The fibrous tunic of the swim bladder of certain fish is composed largely of collagen (ichthyocol) and elastin. Like rat-tail collagen, ichthyocol dissolves to some extent in dilute acetic acid giving very viscous solutions. Freshly removed swim bladders of carp were stripped of their tunics and the latter were purified according to the method of Bergmann and Stein (6). The purified material was suspended in 0.5% acetic acid until a viscous solution had formed which was filtered through a fine sintered glass filter. The absorption curve in the ultraviolet measured with the Beckman spectrophotometer was strikingly similar to that of rat-tail collagen, in that it was essentially lacking in the characteristic absorptions due to phenylalanine, tyrosine, and tryptophan.

Beef tendon collagen offers considerably greater difficulties for spectroscopic studies than does rat-tail collagen. Since beef collagen is relatively insoluble, it was necessary to prepare a colloidal gel using acetic acid solutions. Certain impurities (*e.g.*, mucoproteins) insoluble in the acid were removed, after the gel had been allowed to stand for several days, by filtering through glass wool. In Curve 3 of Fig. 1 is shown the absorption curve of beef tendon collagen (about 0.1% in 5% acetic acid) purified according to Bergmann and Stein (6) by extracting once with Na_2HPO_4. Curve 2 of Fig. 1 represents beef tendon collagen after 3 successive prolonged extractions with Na_2HPO_4. The prominent absorption maxima at 269 and 279 mμ are doubtless due to impurities which are present. It is apparent that successive washings with phosphate, although contributing greatly to the purification of collagen, are only partially effective in removing these contaminants.

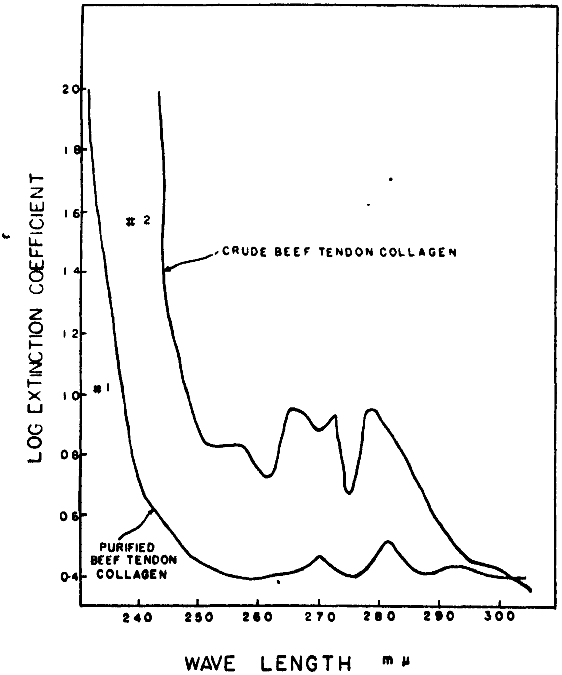

FIG. 2. Ultraviolet absorption spectra of beef tendon collagen in different stages of purification. Curve 1. Film prepared from Na_2HPO_4—extracted, trypsin—treated, collagen. Curve 2. Collagen purified by successive precipitations by Na_2HPO_4 from dilute acetic acid gels.

Beef tendon collagen has also been purified by dispersing it as a homogeneous gel in 2% acetic acid, then precipitating with Na_2HPO_4. The collagen was then prepared as a uniform 0.1% gel in dilute acetic acid. From the absorption of this preparation (Fig. 2, Curve 2) it appears that large amounts of impurities (probably mostly proteins) still remain. Successive suspension in dilute acetic acid followed by reprecipitation with Na_2HPO_4 resulted in removal of a considerable fraction of these contaminants.

In view of the marked refractoriness of native collagen to trypsin, attempts were made to remove impurities from collagen by tryptic digestion. Two g. of beef tendon were pulverized and passed through a No. 20 mesh screen, and then extracted with Na_2HPO_4 in the usual manner. The residue was then digested at pH 7 with 200 mg. of trypsin (Cenco) for 18 hr. at 37°C. The residue was thoroughly washed with distilled water. It was then homogenized in 2% acetic acid, and the gel poured out on a glass plate and permitted to dry. The residual acid was removed by successive washings of the film. The dried film was 25.4 μ thick and, when dispersed in water, gave a pH of 6.05. The film was used directly in the spectrophotometer for measurement of the absorption in the ultraviolet. When dealing with relatively insoluble proteins, the examination of films of the protein has the advantage of yielding a preparation for ultraviolet studies which is relatively transparent and homogeneous and presents to the beam all of the components (both soluble and insoluble) of the original preparation The absorption curve (Fig. 2, Curve 1) indicates the superiority of trypsin treatment plus salt extraction for purifying collagen, but the small maxima in absorption at 270 and 281 mμ indicate that some impurities still remain. It seems likely that beef tendon collagen of a very high degree of purity might be obtained by pulverizing the tendon, successively extracting with Na_2HPO_4, digesting with trypsin followed by water extraction, then succesisvely homogenizing in dilute acetic acid followed by precipitation with Na_2HPO_4.

DISCUSSION

To rate the purity of collagen preparations, it is necessary to have a standard of "pure" collagen. As a tentative standard, purified rat-tail collagen (Fig. 1, Curve 1) may be used. This, like ichthyocol, shows no selective absorption in the region from 257.5 to 287.5 mμ. It will be assumed tentatively, therefore, that true collagen shows only general absorption in this region.

To understand clearly the optical problem of determining impurities in such a preparation, it is necessary to consider the absorption of radiation by mixtures of substances. The absorption of monochromatic radiation by a homogeneous solution is an exponential function of the thickness and concentration of the absorbing layer. If several substances are components of the same solution and do not interact in any way so as to change their individual contributions to the absorption, then the optical density, D, of the solution is:

$$D = \log_{10} \frac{I_o}{I_x} = (k_1c_1 + k_2c_2 + k_3c_3 + \cdots)\, x, \qquad (1)$$

where I_o is the intensity of incident radiation, I_x the intensity of transmitted radiation, k_1, k_2, k_3, *etc.*, the extinction coefficients of component 1, 2, 3, *etc.*, of concentration c_1, c_2, c_3, *etc.*, and x is the thickness of the absorbing layer.

At a particular wavelength, for example 280 mμ, let the optical density ($D = \log_{10}(I_o/I_x)$) attributable to pure collagen be represented by

$$D_c = k_1c_1x_1, \qquad (2)$$

where k_1 and c_1 are the extinction coefficient and concentration, respectively, of the collagen. The optical density of an impure membrane may be expressed as the sum of two terms, one representing the density of pure collagen and the other the density due to impurities.

$$D = D_c + D_1 = [k_1c_1]\, x + [k_2c_2 + k_3c_3 + k_4c_4 + \cdots]x. \qquad (3)$$

For a given membrane, D may be determined by the measured absorption at the wavelength in question, and D_c may be deduced from the experimentally determined absorption curve by drawing a smooth "base line" through the minima of all absorption discontinuities. This assumes that pure collagen has no selective absorption in the region in question. Then

$$p = \frac{D_c}{D} = \frac{k_1c_1}{(k_1c_1) + (k_2c_2 + k_3c_3 + k_4c_4 + \cdots)}. \qquad (4)$$

This expression, when applied to such wavelengths as 280, 270, and 265 mμ, at which selective absorption due to impurities is apt to occur, gives a *factor of merit* for the purity of collagen preparations independent of the thickness or water content of the membranes measured. For pure collagen the factor $P = 1$; for impure collagen $P < 1$.

SUMMARY

The absorption spectra of rat-tail, ichthyocol and highly purified beef tendon collagen resemble closely that of purified gelatin in showing little specific absorption at wavelengths above 240 mμ. Evidence is

TABLE I

P Factors for Collagen Preparations

Preparation	$P = (D_c/D)$		
	265 mμ	270 mμ	280 mμ
Highly purified rat-tail collagen (see Fig. 1, Curve 1).	1.00	1.00	1.00
Membrane from trypsinized beef collagen in 2% acetic acid (see Fig. 2, curve 1).	0.98	0.85	0.80
Beef tendon precipitated once from acid solution (see Fig. 2, Curve 2).	0.74	0.77	0.68
Once-extracted beef tendon dissolved in 5% acetic acid (see Fig. 1, Curve 3).	0.95	0.80	0.67
Thrice-extracted beef tendon dissolved in 5% acetic acid (see Fig. 1, Curve 2).	0.94	0.84	0.59

presented that the slight irregularities and maxima in the spectra of beef-tendon collagen above 240 mμ are due to impurities. It has been possible to use as an index of purity of different collagen preparations the absorption due to contaminants at 265, 270, and 280 mμ. Collagen preparations of high purity have been made by washing beef tendon with phosphate, digesting with trypsin, suspending in acetic acid and precipitating with phosphate.

A new method for the study of the absorption spectra of insoluble proteins is presented. It involves suspension of the protein followed by evaporation of the suspension to a thin film, which is then placed in the path of the light beam.

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Amino Acids as Carbon Source for the Growth of Yeasts

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INTRODUCTION

Definite knowledge on the utilization of the carbon residue of amino acids for the growth of yeasts is limited. In the earlier yeast growth literature before bios factors were identified, natural media were used; consequently, the deductions may not have been justified. Thus, Claassen (1), in comparing yeast yields in natural media, obtained a higher crop when asparagine was added than when ammonium salts were added. He concluded that the higher yield was due to assimilation of the carbon of the amino acid. Wagner (2), on the other hand, attributed this increase in yield to more effective aeration caused by the change in surface tension in the presence of an amino acid. More recently, Sperber (3) and Ehrensvärd *et al.* (4), working with media of known composition, showed that *T. utilis* utilized the carbon of alanine, glycine, and possibly of asparagine in growth and respiration experiments.

Schultz and Pomper (5) reported on the adequacy of individual amino acids as sources of nitrogen for the growth of a number of strains of yeasts in a basal medium of known composition and an adequate supply of dextrose. In the present study, the same basic technique was used to evaluate amino acids as possible carbon sources for building yeast tissue, in the hope that such a study might help solve the mechanism by which yeast synthesizes proteins.

EXPERIMENTAL

The procedure used was essentially that described by Schultz and Pomper (5), in which amino acids were the single source of nitrogen in a medium composed of dextrose, nutrient salts, citrate buffer, and growth factors. The following changes were made in order to determine amino acid carbon utilization.

1. The citrate buffer was replaced by a phosphate buffer.
2. The dextrose content was lowered from the usual 500 mg./10 ml. of final growth medium to that of 10 mg. and to none at all.
3. The amount of amino acid was increased from 4 mg. to 8 mg. with all amino acids but proline, where 14.6 mg./10 ml. of final growth medium was used.

The potassium phosphate buffer was prepared by dissolving 180 g. KH_2PO_4 and 21 g. K_2HPO_4 in distilled water and diluting to a volume of 1 l. This buffer, which had a pH of 5.0, was used at a concentration of 1 ml. in 10 ml. of final growth medium.

Yeast Inoculum

The preparation of the yeast inoculum used in this study has been described previously (6). The inoculation per tube consists of 0.5 ml. of sterile saline solution containing approximately 0.07 mg. moist yeast.

Yeast Growth

The yeasts were grown at 30°C. with shaking, and the extent of growth was measured at 16, 20, 24, and 40 hr. by reading percentage absorption of light on a Lumetron 400 colorimeter fitted for use with 18 mm. O.D. test tubes. A gray glass and wire screen filter was used to reduce the light.

RESULTS

In Tables I and II growth responses are tabulated for two species of *Candida* yeasts, *i.e.*, *pseudotropicalis* and *guilliermondi*. All 18 amino acids that were included in the study on amino acids as nitrogen sources (5) were also used in the study of carbon utilization, but only asparagine and 7 amino acids that showed any promise of being metabolized by the yeast are included in the tables.

Three sets of data are shown under the headings of 500 mg. dextrose, 10 mg. dextrose and no dextrose. Where yeasts were grown in the presence of 500 mg. dextrose, nutrient salts, growth agents and a citrate buffer, it is assumed that only the amino nitrogen and not the carbon residue of the amino acid was utilized by the yeast. Ammonium sulfate or casein hydrolyzate as the only source of nitrogen are included as controls. The rate of growth with either of the latter two nitrogen sources, while in the presence of adequate sugar, was equal to or faster than that of most single amino acids, a fact which was reported by Schultz and Pomper (5).

In the second and third sets of data, yeast growth on amino acid carbon in a medium containing 10 mg. dextrose and no dextrose was compared. A potassium phosphate buffer was substituted for the

TABLE I

Nitrogen and Carbon Utilization of Individual Amino Acids by Candida pseudotropicalis

Nitrogen source	Per cent absorption											
	500 mg. dextrose				10 mg. dextrose				No dextrose			
	16 hr.	20 hr.	24 hr.	40 hr.	16 hr	20 hr.	24 hr.	40 hr.	16 hr.	20 hr.	24 hr.	40 hr.
Alanine	65	88	93	95	45	52	57	78	1	1	1	5
Arginine	72	92	93	94	40	41	41	40	1	2	1	1
Asparagine	57	84	92	94	42	43	43	50	1	1	1	1
Aspartic acid	34	71	84	93	37	45	52	69	2	1	1	4
Glutamic acid	58	84	92	95	57	73	77	87	2	5	8	61
Glycine	2	2	4	35	9	17	17	30	1	1	1	1
Proline	45	81	89	94	69	78	80	86	1	1	3	12
Serine	40	70	83	93	40	52	53	60	1	1	1	1
Ammonium sulfate	78	90	91	92	38	37	37	37	1	1	1	1
Casein hydrolyzate	92	93	93	94								

TABLE II

Nitrogen and Carbon Utilization of Individual Amino Acids by Candida guilliermondi

Nitrogen source	Per cent absorption											
	500 mg. dextrose				10 mg. dextrose				No dextrose			
	16 hr.	20 hr.	24 hr.	40 hr.	16 hr.	20 hr.	24 hr.	40 hr.	16 hr.	20 hr.	24 hr.	40 hr.
Alanine	10	34	63	95	53	61	72	87	3	5	7	50
Arginine	53	81	89	96	52	56	63	79	4	3	4	8
Asparagine	61	85	92	96	48	55	67	83	2	2	7	10
Aspartic acid	16	35	50	93	51	64	74	87	7	13	34	85
Glutamic acid	33	60	76	96	41	62	76	91	4	15	28	89
Glycine	10	25	52	96	14	26	45	76	1	2	1	5
Proline	20	56	75	95	12	42	66	88	7	8	11	35
Serine	25	62	78	93	51	57	67	83	2	2	2	4
Ammonium sulfate	48	80	88	95	44	43	43	42	2	1	2	3
Casein hydrolyzate	71	86	90	95					—			

citrate buffer to eliminate any possible growth on citrate carbon. In the absence of any buffer, it was found that, after full growth on the carbon of an amino acid, the resulting pH rose to about 8.5. In the presence of the buffer, the pH rose to 6.5–7.0. In general, however, the rate of growth was only slightly different with or without a buffer. In the experiments in which there was an absence of dextrose, the yeast had to rely on the nitrogen and the carbon of the amino acid for growth. In the second set of data, the 10 mg. of dextrose allowed the yeast to grow, as shown by the $(NH_4)_2SO_4$ control, to an absorption value of about 40. Any further growth will be at the expense of the amino acid carbon. In the absence of dextrose as a starter, $(NH_4)_2SO_4$ alone showed no growth; therefore, any increase of absorption with single amino acids indicated that the yeast was capable of building yeast tissue from the carbon residue of the amino acid.

Results obtained with the two *Candida* yeasts on 500 mg. dextrose showed that the species *pseudotropicalis* grew at a rate equal to or slightly better than the species *guilliermondi* on all of the amino acids listed, excepting glycine. The former indicated that it was readily capable of deaminating these amino acids. However, the growth of *pseudotropicalis* on either 10 mg. dextrose or in the absence of dextrose was much more restricted. *Pseudotropicalis* was capable, under the conditions of the experiment, of metabolizing the carbon of glutamic acid and proline in the absence of dextrose as a starter, and glutamic acid, proline, alanine, aspartic acid and serine in the presence of 10 mg. of dextrose. The *guilliermondi* yeast, on the other hand, was able to grow on the carbon of 4 amino acids and asparagine and possibly arginine, without dextrose as a starter, and on all 8 compounds (Table II) in the presence of a small amount of dextrose as a starter. Hence, the two species of *Candida* yeasts, although quite similar in their nitrogen utilization of amino acids, exhibited a great difference in their metabolism of the carbon residue of amino acids.

Table III lists the growth of 9 yeast cultures on single amino acids with and without dextrose. The utilization is designated as probable, definite, and good, and is further defined in the legend. The yeasts are arranged in the order of their increasing ability to utilize the amino acids. As was shown with the two *Candida* yeasts, of which complete data are given in Tables I and II and which are repeated in Table III, all yeasts were aided in their utilization of amino acid carbon by the initiation of growth with 10 mg. of dextrose. *Saccharomyces cerevisiae*

TABLE III

Carbon Utilization of Individual Amino Acids by Yeasts

Culture	Bios no.	Dextrose	Glutamic acid	Proline	Alanine	Asparagine	Aspartic acid	Serine	Glycine	Arginine
		mg.								
S. cerevisiae	23	10	?	+	—	—	—	—	—	—
		0	—	—	—	—	—	—	—	—
T. cremoris	237	10	+	+	?	—	—	—	—	—
		0	+	—	?	—	—	—	—	—
Zygosaccharomyces marxianus	7	10	++	++	+	—	—	—	—	—
		0	+	—	—	—	—	—	—	—
Mycoderma[a]	0	10	++	++	++	—	—	—	—	?
		0	++	?	?	—	—	—	—	—
Candida pseudotropicalis	37[b]	10	++	++	++	?	+	+	—	—
		0	+	+	—	—	—	—	—	—
T. pulcherrima	3	10	++	++	+	++	++	+	—	—
		0	+	+	+	+	+	—	—	—
T. utilis	0	10	++	++	++	++	++	++	+	?
		0	++	++	++	?	?	?	?	—
S. pastorianus	3	10	++	++	++	++	++	++	+	++
		0	++	+	+	+	++	+	—	++
Candida guilliermondi	3	10	++	++	++	++	++	++	++	++
		0	++	+	+	+	++	—	—	?

? = Probable utilization.

+ = Definite utilization.

++ = Good utilization, above 70% light absorption with or without dextrose.

[a] An asporogenous film-forming yeast, not classified as to species.

[b] This yeast was previously reported as BN 7.

was able to attack only proline and probably glutamic acid when dextrose was present as a starter. A number of other strains and varieties of yeasts belonging to this species, for which data are not given here, showed the same general inability to attack the carbon of amino acids.

S. pastorianus is able to use any of these 8 compounds as a sole source of carbon when used with 10 mg. dextrose.

In general, yeasts that showed a very high rate of growth when utilizing glutamic acid nitrogen were most likely to show good amino acid carbon utilization. The growth requirements of these yeasts were usually those with bios numbers 0 (requiring no growth factors), 3 (requiring biotin), and 7 (requiring nicotinic acid) (6).

Discussion

Yeasts are better able to initiate and continue growing on the amino acid carbon when using 10 mg. dextrose as a starter than in the absence of sugar. A 2-fold explanation for this starter effect is offered. The sugar furnishes the necessary carbon intermediates used in normal growth, or the well nourished seed yeast from the agar slant is so rich in nitrogen that deamination of the amino acid in the absence of any available carbohydrate is slower than if the yeast were lower in nitrogen content, and thus forced to use nitrogen from the medium. In support of the latter contention, Sperber (3) has noticed a difference in the rate of attack on nitrogen-containing substances by *Torulopsis* yeast of high and low contents of nitrogen. A yeast already high in nitrogen did not attack such nitrogen-containing substances in experiments lasting for 3 to 4 hr. It is logical to assume that, in the absence of a carbohydrate source, the yeast must first deaminate the amino acids before they can be utilized as a carbon source. Some preliminary experiments with certain mono- and dicarboxylic acids seem to indicate that they may exert a specific effect on amino acid carbon utilization.

Saccharomyces cerevisiae yeasts grow poorly on amino acid carbon. The question is whether yeasts that are scarcely capable of growing on amino acid carbon are synthesizing protein by a different set of enzymes or conversely, whether yeasts that are capable of using amino acid carbon under these forced conditions utilize a different set of enzymes when growing normally on adequate sugar and nitrogen. In this connection Sperber (7), using *Torulopsis* yeast, concludes that the enzymes of the citric acid cycle are possibly not normal constituents of the yeast but can be synthesized under certain conditions.

The amino acids, glutamic acid, proline, alanine, aspartic acid, serine, glycine, and arginine, as well as asparagine, are able to serve as a carbon source in yeast growth. With the exception of asparagine, these are all non-essential amino acids in animal nutrition. Possibly of even greater significance in this study is the fact that they all belong to the glucogenic group of amino acids.

In this survey of yeast cultures, the data, without exception, indicate that yeasts that could not metabolize the carbon of glutamic acid or proline were unable to utilize the carbon of any amino acid. Proline is known to be readily converted to glutamic acid by an oxidation reaction (8). It thus appears that, under the conditions of the experiments, glutamic acid plays a very important and possibly a central role in protein synthesis.

In an attempt to explain amino acid synthesis in yeast by transaminases there are studies by Kritzmann (9), Adler *et al.* (10), Cohen (11) and Roine (12). The latter concludes that it seems very probable that glutamic acid is the primary amino acid in protein synthesis.

SUMMARY

1. Nine yeasts, comprising 4 genera, have been tested for their ability to utilize the carbon residue of 18 amino acids for the purpose of proliferation. A basal medium of nutrient salts, growth substances, and potassium phosphate buffer with and without a small amount of dextrose (10 mg.), was used.

2. It was found that the presence of a small amount of dextrose acted as a starter, aiding in the initiation and continuation of growth of yeasts on amino acid carbon.

3. A few of the yeast cultures were able to utilize the carbon of the following compounds when used singly, namely, alanine, arginine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine.

4. Yeasts that could not attack glutamic acid or proline were unable to utilize the carbon of any amino acid.

5. Under the conditions of the experiments, glutamic acid seems to play an important role in protein synthesis.

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Factors Influencing the Response of the Bioluminescent Reaction to Adenosine Triphosphate[1]

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INTRODUCTION

Light emission by living organisms is the product of a series of chemical reactions. However, it is possible, under certain conditions, to extract from luminous organisms a relatively heat stable substance, *luciferin*, and a heat labile substance, *luciferase*, which, when mixed, will emit light (see 7). Harvey and associates have studied extensively the kinetics of this reaction using a partially purified preparation obtained from the luminous crustacean, *Cypridina hilgendorfii*. These studies demonstrated that the light emitting step depends upon the oxidation of the luciferin molecule in the presence of both oxygen and the enzyme, luciferase. In air, but in the absence of the enzyme, luciferin is slowly oxidized without light emission. This latter oxidative step can be reversed by numerous reductants, while the products of the luminous oxidation fail to respond to these simple hydrogen donors.

The degradation of a ketohydroxy side chain on the luciferin molecule has been suggested as a possible explanation of this irreversible luminescent oxidation (3). Since it was subsequently shown that partially purified *Cypridina* luciferin contained labile phosphate groups which were removed during the light emitting reaction, it has been postulated that the energy derived from the breakdown of the side chain is conserved as phosphate bond energy (9). This suggestion was made to account not only for the irreversible reaction observed *in vitro* but also for the energy requirements of luminescence.

Support for the suggestion that labile phosphate groups are concerned in the luminescent reaction has been obtained recently with extracts from firefly material. It was possible to restore light emission in those extracts which had ceased to luminesce by adding adenosine triphosphate (11). Extracts from whole fireflies which had been stored in the dried state for several months fail to respond to the addition of ATP, although a bright luminescence is obtained upon initially grinding the organism in the presence of water. These results demonstrate that it is possible to keep the fireflies in a dried state and still preserve an active substrate and enzyme, although the system concerned with the utilization of ATP in light production has been destroyed. The results are entirely comparable to those obtained with the dried *Cypridina* from which an active substrate containing labile phosphate (luciferin) and an enzyme (luciferase) have been isolated.

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The present report is concerned with some of the physiological and biochemical properties of the reactions utilizing ATP in the luminescent reaction. The present results demonstrate that the classical luciferin of fireflies can be separated into at least three separate components.

MATERIALS, METHODS, AND RESULTS

Partial Purification of the Luminescent System

As reported previously, it is necessary that fresh material be used for the preparation of active extracts; therefore, in all experiments reported in the present paper, live fireflies (*Photinus pyralis*), collected in the Baltimore area, were used. The live fireflies were placed in a vacuum desiccator containing $CaCl_2$ and were dried overnight under vacuum. Immediately after drying, the abdominal regions containing the light organs, were removed from the fireflies and subsequently ground with purified sand to a fine powder. This powder was placed in a stoppered flask and stored at −18°C. Material prepared and stored as described above has remained active for over a year.

The luminescent system in the fireflies is very soluble in water. The entire luminescent system cannot be adsorbed by any one adsorbent to an extent useful for purification although certain of the proteins necessary for light emission can be taken up by permutit, charcoal (Norit) and alumina. The system is quite sensitive to temperature, being completely inactivated in less than 1 min. at 80°C. On the other hand, the crude preparations appear to be fairly stable to pH changes with very little loss of activity over a period of 1 hr. at 25°C. within the pH range of 4.5–10.0. Sterile aqueous solutions may be preserved for several weeks at 8°C. and for at least a year at −18°C.

Several procedures have been used for partial purification of the luminescent system but the one giving the most consistent results involves fractionation with $(NH_4)_2SO_4$. To obtain the active extract, 4 g. of vacuum-dried firefly lanterns were ground with sand and extracted twice with a total volume of 100 ml. of H_2O. The pH was adjusted with HCl to 6.0, and the debris and precipitate were centrifuged and discarded. All of the light emitting system remained in the supernatant, to which were added 10 g. of

$(NH_4)_2SO_4$. After 15 min. at 0°C., the solution was centrifuged and the inactive precipitate discarded. The supernatant was adjusted to pH 7.5 and an additional 10 g. of $(NH_4)_2SO_4$ added. The solution was again cooled to 0°C. for 15 min. and the inactive precipitate formed was discarded. The supernatant was adjusted to pH 4.5 and 30 g. of $(NH_4)_2SO_4$ added. After cooling, the solution was filtered and the active precipitate dissolved in 50 ml. of water or 1/15 *M* sodium phosphate buffer at pH 7.5. The final preparation has a clear amber color.

Although no light could be obtained from the discarded fractions upon the addition of ATP, the final preparation showed considerable loss of activity during the purification. The activity could be completely restored, however, by the addition of crude aqueous extracts either from the "lanterns" of the fireflies or the non-luminescent bodies. The stimulatory effect of the body extracts could be duplicated by the addition of one of several inorgainc ions, in particular Mg^{++}, Mn^{++} and Co^{++}. The results of these studies are recorded in Table I.

TABLE I

Effect of Inorganic Ions on Luminescence

1 ml. of the purified enzyme system was added to an Na_2HPO_4 buffer containing the appropriate concentration of inorganic ion. ATP was added to give a final concentration of 0.08 mg./ml. The final volume of the reaction mixture was 7 ml.[2]

Compound added	Concentration	Maximum light intensity	Compound added	Concentration	Light intensity
	M	*volts*		*M*	*volts*
None	—	7.0	$CoSO_4$	1×10^{-3}	80.0
$MgSO_4$	1×10^{-4}	50.0	$CoSO_4$	2×10^{-3}	74.0
$MgSO_4$	1×10^{-3}	90.0	$FeSO_4$	1×10^{-3}	21.0
$MgSO_4$	2×10^{-3}	110.0	$FeSO_4$	2×10^{-3}	32.0
$MnSO_4$	3×10^{-4}	32.0	$NiCl_2$	1×10^{-3}	26.0
$MnSO_4$	1×10^{-3}	90.0	$ZnSO_4$	1×10^{-3}	15.0
$MnSO_4$	2×10^{-3}	92.0			

Calcium ions have a slightly inhibitory effect in concentrations equivalent to the above ions. In higher concentrations the inhibition is marked but can be reversed by Mg^{++}.

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[2] The light intensity was measured by an electron multiplier tube (G. E. 931A) whose response and characteristics have been described previously (10). The adenosine triphosphate was prepared from rabbit muscle as the barium salt after two mercury precipitations, according to the usual procedure (see 12).

Relation of Light Intensity to ATP Concentration

Determinations of the amount of adenosine triphosphate required to give maximum light intensity were made in 1/15 *M* sodium phosphate buffer at pH 7.5. The reactions were carried out in a test tube placed directly in front of a phototube. One milliliter of the enzyme system was added rapidly to the phosphate solution containing the appropriate amount of ATP. The final volume of the reaction mixture was in all cases 10 ml. Readings were made every 15 sec. for a period of several min. The relation between maximum light intensity and ATP concentration is shown in Fig. 1. Half saturation of the system is reached at an ATP concentration of approximately 5×10^{-5} *M*. The hydrolysis of ATP in 1 N acid at 100°C. for 12 min. removes all light stimulating qualities.

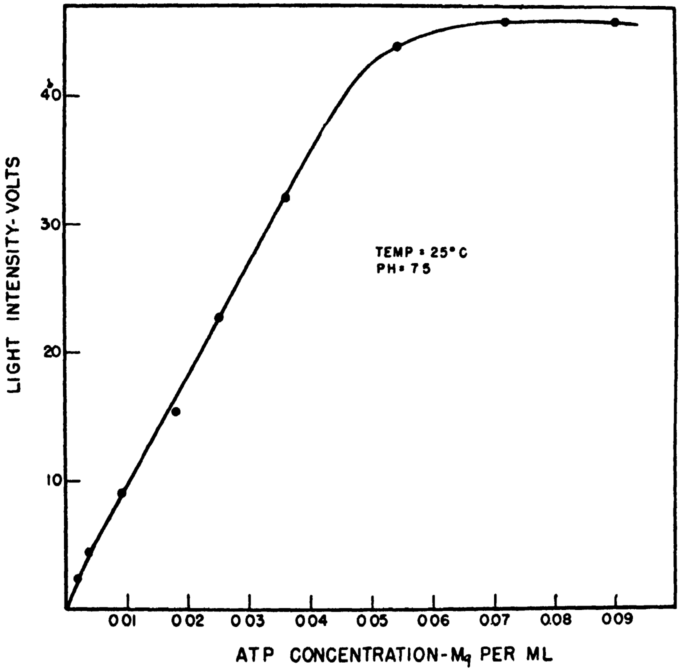

FIG. 1. Relation of light intensity to ATP concentration. One ml. of the enzyme system was mixed with 1/15 *M* Na_2HPO_4 buffer and the appropriate concentration of ATP to give a final volume of 10 ml. See test for details.

Attempts to stimulate light production by reagents other than ATP have failed. Neither creatine phosphate nor creatine phosphate plus adenylic acid is effective nor are various reducing agents such as sodium hydrosulfite, and reduced coenzyme I. At the present time, arginine phosphate has not been tested.

In a previous publication (11) it was noted that the duration of light emission depended upon ATP concentration in much the same way as light intensity depends on ATP. These experiments were done with the crude aqueous extract which has a very high ATPase activity.[3] In the purified preparations, however, the ATPase activity has greatly decreased, so that, although the intensity of luminescence is low, the duration is much greater. With the addition of the inorganic metallic ions the ATP disappears rapidly due to the restoration of ATPase activity. The rate of return of light intensity to the basal level parallels the rate of ATP disappearance. The inorganic ions which are most effective in restoring ATPase activity are also the most effective in giving an initial high light intensity. Since the total light emitted above the basal level for a given concentration of ATP varies with the particular ion, it is evident that the ATP is utilized in systems other than

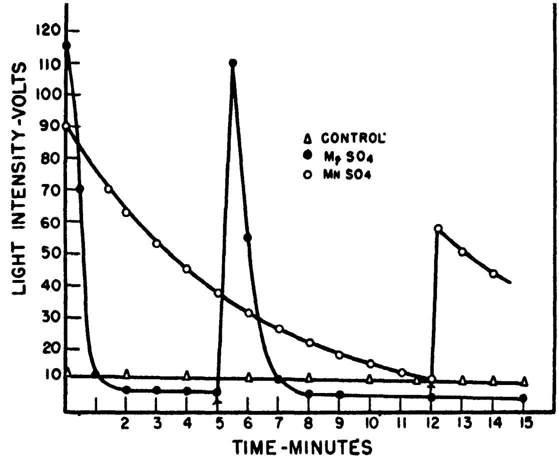

FIG. 2. Relation of light intensity to ATP and inorganic ions. Reaction mixture contained 1 ml. of the purified enzyme preparation and 1/15 *M* Na_2HPO_4 (pH 7.5) to give a final volume of 6 ml. after all components had been added. $MgSO_4$ and $MnSO_4$ were added to give a final concentration of 10^{-3} *M*. At zero time ATP was added to give a final concentration of 0.07 mg./ml. Light readings were made at the appropriate intervals until the intensity returned to the basal level. The arrows indicate that time at which an additional quantity of ATP was added. See text for details.

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[3] One ml. of the crude extract of the firefly lanterns in 4 ml. of ATP-succinate mixture at pH 6.5 will remove in 15 min. approximately 50 γ of phosphate. Luminescence persists until the equivalent of 2 phosphate molecules are removed from the ATP.

those directly concerned in light production. This effect is graphically illustrated in Fig. 2. In these reactions, which were carried out as above, the ATP concentration was sufficient to give maximum light intensity (0.07 mg./ml.). Although Mg^{++} stimulates luminescence considerably, the amount of light obtained for a given concentration of ATP is greatly reduced. In the above experiment almost all of the ATP had disappeared in 1 min. When more ATP was added, the light intensity once again increased, but not entirely to the original level. With Mn^{++}, a given concentration of ATP maintained a high light intensity for a much longer period of time. If additional ATP is added after the light returns to the low basal level, there is a stimulation, but it is far below that originally obtained. The amount of stimulation obtained on the second addition of ATP depends upon the initial amount of ATP added, *i.e.*, the greater the amount of light emitted initially, the less the stimulation upon a second addition of ATP.

From this it can be concluded that an unknown factor is being exhausted when luminescence is maintained for a considerable time. A dialyzate of the crude aqueous extract of lanterns will stimulate those reaction mixtures which have become deficient with long periods of luminescence in the presence of inorgainc ions and an excess of ATP. The non-luminescent portion of the fireflies does not contain this factor. Attempts are being made to purify and identify this component.

It is interesting that, in the above reactions to which inorganic ions have been added, the light intensity does not return completely to zero. This is in contrast to the crude extracts, which rapidly dephosphorylate all of the ATP, at which time the light is extinguished. In the purified preparations it appears likely that the ATP has initiated reactions which will maintain a low luminescence.

Effect of pH and Buffers on Luminescence

Using the same general techniques already described, the pH optimum and the effect of various buffers on the luminescent system have been investigated. In all cases, an ATP concentration was used which was sufficient to give maximum light intensity. Readings were made 30 sec. after 1 ml. of purified enzyme preparation was mixed with the ATP-buffer solution. All pH readings were made with a Beckman pH meter immediately after the light intensity reading was made. The effect of various phosphate buffers at different pH's on the light intensity is shown in Fig. 3. The final molar concentration of the buffers was the same in all cases. The light intensity in the sodium phosphate buffer is approximately 75% higher than in the potassium phosphate buffer. The optimum pH in all cases is approximately 7.75, although, in the

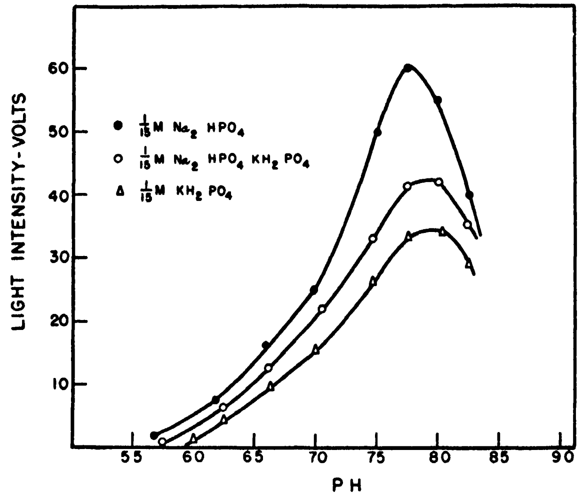

FIG. 3. Effect of pH and buffers on luminescence.

presence of K^+ the optimum appears to be shifted slightly to the alkaline side. Succinate gives a pH activity curve similar to the phosphates but is slightly better for light production than the K^+ phosphate buffer.

The results obtained with the sodium and potassium buffers differ, in many respects, from those obtained by Chase (4) on a *Cypridina* preparation. In the latter studies, the velocity constant of the reaction was much greater in K^+ or Na^+ buffers than in a mixture of the two. The contrasting effects of these buffers probably represent a difference in the site of action of the K^+ or Na^+ ions in the extracts of the two organisms, at least as far as the rate-controlling reaction is concerned. The difference may be related to the action of these ions on the ATPase activity.

Temperature Optimum

The temperature optimum for luminescence was determined in a sodium phosphate buffer (pH 7.5) at an ATP concentration sufficient to saturate the system. To insure constant luminescence throughout the experiment no inorganic elements were added. The results are plotted in Fig. 4. The temperature optimum is 25°C. under these conditions. With ATP concentrations insufficient to saturate the enzyme, the rate remains the same from approximately 18°C. to 30°C. A plot of the log of light intensity against the reciprocal of the absolute temperature is shown in Fig. 5. The slope of the line gives a value for the energy of activation ($\Delta H^{\pm}$) equal to 18,500 calories, a value which agrees with that obtained in luminous bacteria (See Harvey '40).

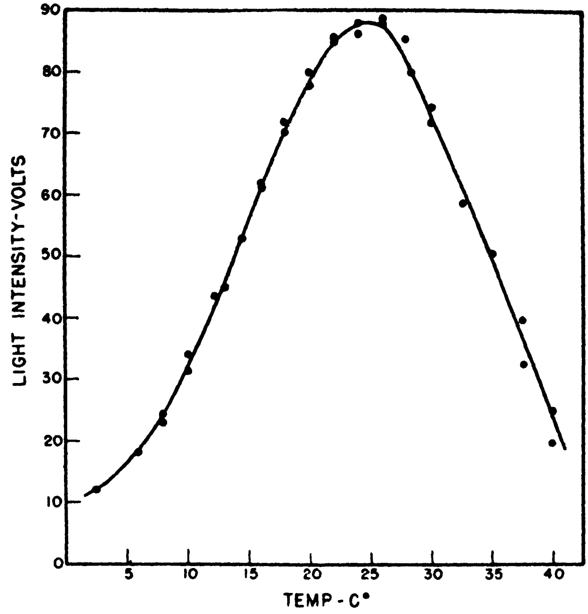

FIG. 4. Relation of light intensity to temperature.

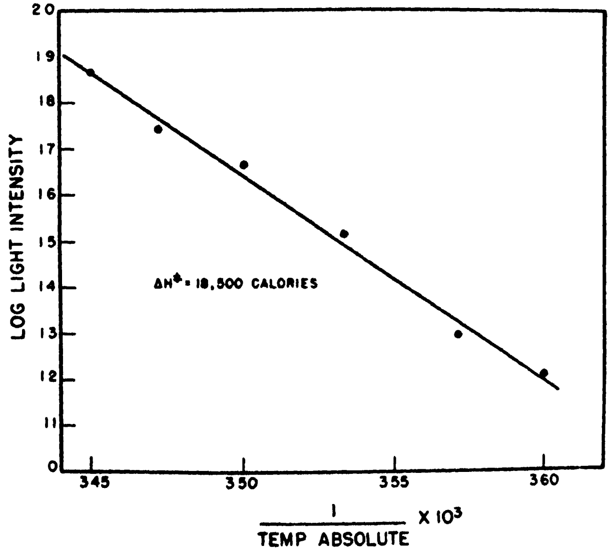

FIG. 5. The log of light intensity as a function of the reciprocal of the absolute temperature. Data from Fig. 4.

Effect of Inhibitors

It was previously reported that oxygen was necessary for light production in the crude extracts of fireflies when ATP was added. Since the oxygen consumption in the luminescent reaction in bacteria and other forms is cyanide-insensitive, it was of interest to determine whether or not the oxidative luminescent reaction which takes place in the presence of ATP is also cyanide-insensitive. The results of these and other studies are presented in Table II. Not only does cyanide fail to inhibit luminescence but actually stimulates it to a considerable extent. This phenomenon has also been observed in luminous bacteria.

TABLE II

Effect of Inhibitors on Luminescence

One ml. of the purified luminescent system was mixed with 5 ml. of 1/15 *M* Na_2HPO_4 buffer containing $MnSO_4$, 1.4 mg. ATP/ml. and the appropriate amount of inhibitor to give the final molar concentration as shown in the table. Readings were made 30 sec. after mixing.

Inhibitor	Concentration	Inhibition	pH
	M	*per cent*	
2-Methyl-1,4-naphthoquinone	3.0×10^{-6}	26	6.5
2-Methyl-1,4-naphthoquinone	5.5×10^{-6}	53	6.5
Sodium azide	1.0×10^{-3}	5	7.4
Sodium azide	2.0×10^{-3}	22	7.4
NaCN	1.0×10^{-3}	0	7.4
NaF	1.0×10^{-2}	12	7.4
Urethane	0.1	48	7.4

In previous studies dealing with napthoquinone inhibition of luminescence, it was postulated that cyanine would also stimulate under conditions where reducing substances were limiting (10). It seems possible that, in the present system, cyanide, by inhibiting the cytochrome system, stimulates the light emitting reaction by increasing the effective electron pressure. The largest stimulation obtained with cyanide thus far has been 20%.

In general the degree of inhibition obtained with other agents agrees with their effects on the luminescent reaction *in vivo*.

Emission Spectrum

The spectral energy distribution of the light emitted by the firefly extracts in the presence of ATP has been determined.[4] The emission curve was found to be unsymmetrical, extending from 470 mμ to 700 mμ, with a maximum at 565 mμ. By plotting the spectral energy against frequency it was possible to demonstrate two fundamental frequencies, one at 17,630 cm^{-1} and the second at 16,200 cm^{-1}. Eymers and van Schouwenburg (6), using the data of Coblentz and Hughes (5), demonstrated that these two fundamental frequencies were also present in the light emitted by the intact firefly. The above results indicate that the structural components of the firefly have little to do with the nature of the emitted light, the emission curve being characteristic of the chemical components of the light reaction itself.

Many investigators have pointed out that the peak of the emission spectrum of firefly light corresponds to the peak of the fluorescence spectrum of riboflavin and its derivatives (see 7). It is significant, therefore, that both Brooks (2) and Ball and Ramsdell (1) have shown that the lanterns of fireflies contain large quantities of riboflavin. Unfortunately, it is not possible at the present time to state definitely the role of flavin derivatives in the luminescent process (see 8).

Discussion and General Conclusions

From these experiments, certain conclusions can be drawn concerning the mechanism of luminescence of firefly extracts. The existence of an intimate relationship between ATPase activity and luminescence, already discussed in a previous paper, has been further substantiated by the finding that, in those purified preparations described, the failure of the luminescent system to respond to ATP is paralleled by a corresponding loss of ATPase activity. Conversely, when any one of a number of divalent ions (Mg^{++}, Co^{++}, or Mn^{++}) is added to a partially purified extract, luminescence as well as ATPase activity is restored.

A comparison of the effects of the various divalent ions shows, as might be expected, that ATPase activity concerned with the luminescent reaction is not the sole ATPase activity in the extract. The differential stimulation of light production (total luminescence) by Mg^{++}, on the one hand, and Mn^{++} and Co^{++} on the other hand, can be interpreted in the following manner. Mg^{++} apparently stimulates both the luminescent and non-luminescent ATPase systems while Mn^{++} and Co^{++} seem to favor the utilization of ATP by the light producing system. The fact that total light emission is greater at higher Mn^{++} concentrations indicates that this ion in appropriate concentrations actually inhibits the non-luminescent ATPase activity while stimulating its luminescent counterpart.

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[4] McElroy, W. D., and Rainwater, C. S., *J. Cellular Comp. Physiol.* in press.

During the examination of the effects of these ions on light production, it was found that the response obtainable on the second addition of ATP varied inversely as the total light emitted by earlier addition of ATP. This was taken to mean that an unidentified factor was becoming limiting in luminescence. Striking confirmation of this was obtained by the fact that a dialyzate of the crude firefly extract, when added to the "exhausted" preparation in the presence of ATP and Mn^{++} ions, restored the initial light intensity of the system. This demonstrated that the limiting factor was not an enzyme concerned in the reaction, or one of the ions used, but rather, a diffusible compound witha a low molecular weight. Thus far, it has been impossible to add any identified factor to the exhausted system which will restore light. All the known vitamins, amino acids, reduced coenzyme I, yeast extract, hydrolyzed casein and $Na_2S_2O_4$ were without a positive effect. Attempts are being made to isolate and identify this limiting component. Two fluorescent compounds of the firefly, luciferesceine and a riboflavin derivative, are incapable of restoring luminescence when added to the exhausted preparation.

Attempts to separate the purified luminescent system into a dialyzable portion and a non-dialyzable portion have been unsuccessful. After 24 hr. dialysis, the enzyme has lost its ability to respond to ATP. Activity cannot be restored by the addition of the dialyzate. It was thought that perhaps SH groups were becoming oxidized during dialysis, but the addition of cysteine to the reaction mixture did not restore luminescence. Inasmuch as riboflavin is present in high concentrations in the firefly tail, it was considered possible that loss of activity was due to the separation of a riboflavin-containing prosthetic group from the protein moiety. Riboflavin was, however, found to be incapable of stabilizing the enzyme on dialysis.

Previous studies (see 7) on the luminescent system of fireflies indicated that extracts in which the enzymes were inactivated before utilization of the luciferin, when added to other extracts which had ceased to luminesce, would cause some stimulation. It now appears likely that more than luciferin, or something other than luciferin, was added from these extracts. The above results indicate that at least two known factors, inorganic ions and ATP, as well as an unknown (luciferin) factor could stimulate light emission under the described conditions. Furthermore, the previous observations concerned with the inactivation of the light system by dialysis and its restoration by addition of the dialyzate may be interpreted as being due to a loss of inorganic ions and possibly some ATP.

Although evidence as to the general significance of the luminescent system in the metabolism of the organism is incomplete, a number of highly suggestive facts are already known. Harvey (7), in speaking of the evolution of luminescence, has suggested that some oxidative pathway has been altered in such a way that its intermediates may become excited through oxidation and subsequently "decompose" in such a way as to give off visible radiation. The fact that purified *Cypridina* luciferin contains acid-labile phosphate, as well as the finding that ATP stimulates luminescence in firefly extracts, indicates that the luminescent system is involved in both phosphate and electron transport. In tissue extracts it is possible, as the above experiments demonstrate, to transfer the energy of ATP to some system concerned with light production. It is possible that the phosphate acceptor in this case is the luciferin molecule. Of general interest in this connection is the fact that extracts of whole fireflies which have been kept in the dried state for several months fail to respond to the addition of ATP, although a brilliant light is obtained on grinding these dried organisms with water. It is apparent that the system coupling ATP to the light reaction has been lost, while the active luciferin which was present when the fireflies were dried has remained stable. As mentioned previously, the results are entirely comparable to those obtained with the active extracts prepared form dried *Cypridina*, in which it has been demonstrated that labile phosphate is present. It is to be expected, therefore, that the stable component present in the dried whole fireflies also contains labile phosphate groups. The combination of this reduced phosphate ester with its oxidant (oxygen) in the presence of the specific enzyme, luciferase, possibly leads to the instability of the energy-rich complex with subsequent light emission in a certain percentage of the molecules.

Whether energy-rich phosphates for the light producing reaction in the intact organisms are derived from ATP as in the extracts, or whether they are derived from some oxidative process not necessarily involving the adenylic acid system directly, is at present impossible to answer. Previous studies have indicated that the light system competes with the cytochrome system for electrons derived from reduced intermediates. It appears possible that phosphate bond energy may be evolved in this oxidation-reduction reaction and used directly in the light emitting process.

ACKNOWLEDGMENT

We would like to thank Dr. Francis Haxo for his suggestions and criticisms during the preparation of this manuscript.

SUMMARY

1. A procedure for the partial purification by $(NH_4)_2SO_4$ fractionation of firefly extracts which will emit light upon the addition of ATP has been described.

2. Luminescent activity lost during purification of the system may be restored by the addition of one of several different inorganic ions. Mg^{++}, Mn^{++} and Co^{++} are most effective.

3. The inorganic ions which are most effective in restoring luminescence are also the most effective in restoring ATPase activity.

4. In such cases the initial light intensity, as well as the total light emitted, depends upon the quantity of ATP added as well as the particular inorganic ion used. The rate of decay of light intensity in the presence of the inorganic ions parallels the rate of ATP disappearance. The return of light intensity to a low basal level is most rapid with Mg^{++}.

5. During luminescence in the presence of excess ATP and inorganic ions an additional, unidentified, dialyzable factor becomes limiting. This factor is present only in the light organs.

6. In sodium phosphate buffer the optimum pH of the luminescent reaction is 7.5. In a potassium phosphate buffer, the light intensity is depressed. Other buffers were also studied.

7. The temperature optimum of the reaction is 25 C. and the energy of activation ($\Delta H^{\pm}$) is 18,500 calories.

8. Cyanide and azide have very little effect on the luminescent reaction while 2-methyl-1,4-naphthoquinone is quite inhibitory.

9. The emission spectrum of the purified extract is not appreciably different from that of the whole firefly, showing a maximum at 565 $m\mu$.

10. These results are discussed and the question of the origin of the energy-rich phosphates required for luminescence in the organism as compared to the extracts is considered.

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Adenine as a Growth Factor for Etiolated Peas and its Relation to the Thermal Inactivation of Growth

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INTRODUCTION

If sub-apical sections of etiolated pea epicotyls are excised and placed in aqueous media containing sufficient quantities of sucrose, phosphate buffer and indoleacetic acid (IAA), they will grow rapidly, increasing their length 70–80% in 24 hr. (1). Since the results of such growth tests are highly reproducible from day to day, these sections constitute a desirable material for the study of the mechanism of the growth process in plants. In the present work we have investigated the following question: When adequate sucrose, phosphate buffer and IAA are supplied to pea sections, what factor becomes limiting to their growth in length? Information obtained from such a study might be expected to shed light not only upon the biochemistry of growth *per se*, but also upon the specific manner in which auxin exerts its effect upon plant growth. Experiments of this general nature have recently been conducted upon *Avena* coleoptile sections by Bonner (2), who concluded that, once the requirement for auxin is satisfied, growth is effectively limited by arginine. In the experiments described below, evidence is presented which indicates that adenine and related compounds occupy the same role in the growth of the pea plant that arginine does in the *Avena* plant.

MATERIALS AND METHODS

A detailed account of procedures employed has already been reported elsewhere (1), so that only a brief statement concerning materials and methods will be made here.

Alaska peas were soaked in tap water for 2 hr. and then sown in flats containing washed river sand. They were allowed to germinate and grow in a dark room whose temperature was 25°C. The only light to which they were exposed prior to harvest was phototropically unreactive light transmitted by an orange-red Corning filter (No. 348).

At the age of 7–8 days after planting, the etiolated pea epicotyls were about 20 cm. tall and had three nodes on the erect portion of the stem. The two lower nodes produce scales which are morphologically etiolated stipules. In the axils of these scales are found one or more buds which customarily remain dormant. For most of the experiments on bud growth herein described, the experimental material consisted of decapitated 10 cm.-long sections of the epicotyl, containing scale node No. 2 in the median third of the section. Such epicotyl sections were, immediately after cutting, placed with their bases in tap water, and after several hours groups of 12 were transferred to 50 ml. Erlenmeyer flasks containing 10 ml. of the solution to be tested. The orifice of the flask was closed by inserting a small cotton plug against the upper ends of the epicotyls. Experiments were usually conducted over a period of about 7 days. To minimize the effects due to microbial contamination of the test solutions, the solutions were changed every 2 or 3 days. To provide unambiguous confirmation of certain of the results, sterile culture techniques were employed.

Flasks containing the experimental epicotyl sections were either allowed to remain in the dark room or were removed to a light chamber also kept at 25°C. Light of about 500 ft.-candles intensity was supplied by batteries of 40 watt "Daylight" and "White" Mazda fluorescent tubes.

In experiments involving the growth of the normally dormant buds of node No. 2, two types of measurements were made: (a) The length of each bud produced was measured, after which group averages and standard errors were computed. (b) The buds were excised and weighed individually or in groups.

In the experiments on root initiation, the epicotyl sections were treated as above, with one additional manipulation. It is difficult to introduce indoleacetic acid (IAA) into plant materials from the base, because of the well-known basipetal polar translocation of this substance (3). One may reverse this polarity only by the use of high concentrations of IAA applied at the base. To keep the IAA treatments as close as possible to physiological levels, all material treated with this hormone was inverted for 24 hr. in the test solution containing IAA, and was then reinverted and placed in IAA-free sucrose solutions buffered at pH 6.1 by $M/60$ phosphate buffer. After 7 days, numerous roots and root primordia were easily discernible macroscopically. These were counted to give a measure of the root-initiating potency of the test solution.

For experiments involving the growth of sections of the epicotyl, 5 mm. long subterminal sections of the third internode were used. These were conveniently obtained by decapitating the epicotyls, inserting them into a "coleoptile microtome" (1) and excising standard lengths by means of a razor blade. Groups of 12 sections were immersed in 5 cc. portions of test solution contained in 50 ml. Erlenmeyer flasks. Growth measurements were made after 16–24 hr. Details concerning the growth characteristics of such sections have been previously reported (1).

RESULTS

A. *Experiments on the Growth of Epicotyl Sections*

In order to screen compounds for their possible effect on the growth rate of pea epicotyl sections, the test materials were incorporated into media containing 2% sucrose and 0.1 mg./l. IAA buffered at pH 6.1 by $M/60$ phosphate buffer. If any tested compound gave indications of exerting either a stimulatory or inhibitory effect upon growth, its activity was then further investigated over a wide range of concentrations.

For the initial tests, inorganic salts were added at a concentration of 100 mg./l., except for the micronutrients, which were added as 0.1 cc. of the Haas-Reed "A-Z" solution/5 cc. of growth medium. Amino acids, amides, and organic acids were initially tested at a concentration of 50 mg./l., and vitamins and nucleic acid constituents at concentrations ranging from 0.1 to 10 mg./l. Although several of the amino acids gave consistent growth stimulations at concentrations of 30–300 mg./l., much smaller concentrations of adenine and related compounds produced a significantly greater enhancement of growth. It was therefore concluded that, under the conditions of these experiments, adenine was the factor most limiting to the growth of the sections. A generalized summary for such experiments is presented in Table I; a more detailed summary for the growth effects of adenine and related compounds is presented in Table II.

During subsequent experiments, it was fortuitously discovered that the response to exogenous adenine is even greater at higher temperatures. As has been previously described (1), these pea epicotyl sections show a rather sharp temperature optimum for growth at about 30°C., there being a marked inhibition of growth at temperatures of 35°C. and higher. If sections are grown at these elevated temperatures, the thermal inhibition of growth may be largely overcome by the addition of relatively small quantities (about 5–10 γ/cc.) of adenine to the medium (Fig. 1). This would indicate that the capacity of the pea plant to produce sufficient adenine for growth is reduced at elevated temperatures.

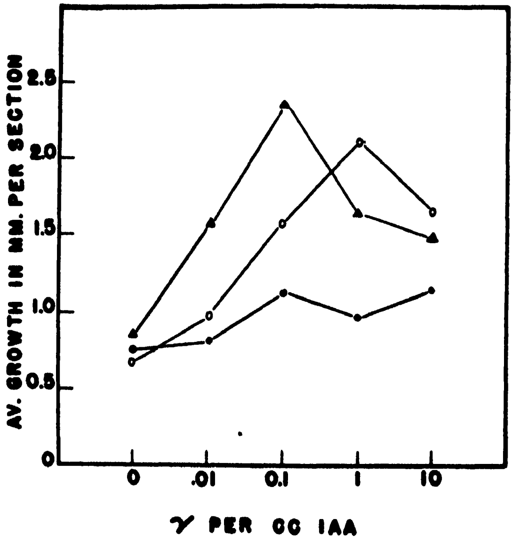

FIG. 1. The effect of adenine on the thermal inactivation of growth. 12 sections immersed in 5 cc. of test medium, and allowed to grow for 16 hr. in the dark at the indicated temperature. ▲ Control solution (2% sucrose, pH 6.1 phosphate buffer); 30°C. ● Control solution; 35°C. ○ Control solution plus 5 γ/cc. adenine; 35°C.

TABLE I

The Effect of Various Classes of Compounds on the Growth of Etiolated Pea Epicotyl Sections Supplied with 2% Sucrose, 0.1 mg./l. IAA and M/60 pH 6.1 Phosphate Buffer

Twelve sections were immersed in 5 cc. of test solution; temperature = 25°C., measurements made after 16 hr.

Class of compounds	Specific compounds tested	Concentrations employed	Growth effect
Inorganic salts	KNO_3; $MgSO_4$; $Ca(NO_3)_2$; $(NH_4)_2SO_4$ A-Z trace element solution	100 mg./l. 0.1 cc./5 cc.	Essentially none
Amino acids and amides	DL-α-Alanine, β-alanine, L(+) arginine, DL-aspartic acid, citrulline, L(−) cystine, L(+) glutamic acid, glycine, L(−) histidine, L-hydroxy-proline, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-norleucine, DL-norvaline, DL-ornithine, DL-β-phenylalanine, L(−) proline, DL-serine, DL-threonine, L(−) tryptophan, L(−) tyrosine, DL-valine, asparagine, glutamine, betaine, urea.	50 mg./l.	α-Alanine, serine, threonine, arginine, asparagine and urea gave up to 25% growth stimulation.
Organic acids	Acetic, succinic, fumaric, citric, pyruvic, oxalacetic, malic and α-ketoglutaric acids.	50 mg./l.	About 20% growth stimulation by succinic acid.
Vitamins	Thiamine, riboflavin, pyridoxine, nicotinamide, Ca pantothenate, biotin, inositol, p-aminobenzoic acid, choline, folic acid, ascorbic acid, ergosterol, 2-methyl-1,4-naphthoquinone.	0.1-10 mg./l.	Growth inhibition by riboflavin in the light.[a]
Nucleic acid constituents	Adenine, adenosine, adenylic acid, guanine, guanosine, guanylic acid, hypoxanthine, xanthine, uric acid, cytosine, cytidylic acid, uracil.	0.1-10 mg./l.	Marked stimulation by adenine and related compounds; slight stimulation by uracil.

[a] This has been shown to be due to a riboflavin-sensitized photoöxidation of indoleacetic acid (17).

TABLE II

The Effect of Adenine and Related Compounds on the Growth of 5 mm. Subapical Sections of Etiolated Pea Epicotyl

Experiment conducted in darkness, temperature 25°C., 12 sections placed in 5 cc. of the test solution contained in a 50 cc. Erlenmeyer flask.

Expt. no.	Growth solution	Average growth in mm./section	Relative growth
P-52	2% Sucrose+pH 6.1 phosphate buffer (control)	1.02±0.19[a]	100
	Control+10 γ/cc. adenine	1.28±0.21	121
	Control+0.1 γ/cc. IAA	2.03±0.10	192
	Control+0.1 γ/cc. IAA+10 γ/cc. adenine	2.99±0.14	282
P-168	Control	1.00	100
	Control+5 γ/cc. adenine	1.07	107
	Control+1 γ/cc. IAA	2.46	246
	Control+1 γ/cc. IAA+5 γ/cc. adenine	3.39	339
P-172	Control	1.19	100
	Control+1 γ/cc. IAA	1.89	159
	Control+1 γ/cc. IAA+10 γ/cc. adenosine	2.22	187
	Control+1 γ/cc. IAA+10 γ/cc. adenylic acid	2.31	194

[a] Standard error.

It is interesting to note that certain "adenineless" mutants of *Neurospora* are temperature-sensitive; that is, they are able to synthesize adequate adenine for their growth needs at temperatures of 28°C. or lower, but require exogenous adenine when grown at elevated temperatures (4). Although our experiments have not adequately distinguished between decreased adenine production and enhanced adenine destruction at the elevated temperatures, it would seem that there is a parallelism between the response of etiolated peas and the temperature-sensitive adenineless *Neurospora* mutant.

B. Experiments on the Growth of Lateral Buds

D. Bonner, Haagen-Smit and Went (5) demonstrated that a sterile diffusate of pea cotyledons was capable of stimulating the growth of the leaves of pea and other plant species. Subsequently, Bonner and Haagen-Smit (6) reported that adenine and other purines could duplicate this effect, and finally D. Bonner (7) was able to isolate hypoxanthine from the pea diffusate. In subsequent experiments, Bonner and Bonner (8) claimed that adenine greatly stimulated leaf growth in intact *Cosmos* plants, but Kruyt and Veldstra (9) were unable to confirm their findings. De Ropp (10) also failed to find any effect of adenine on the growth of leaves of cultured stem tips of rye. In a paper which appeared after the completion of the present work, Skoog and Tsui (11) report that adenine induces bud formation in callus and normal tobacco tissues grown in pure culture. In view of these reports, and in view of the fact that adenine was known to stimulate the growth of our etiolated pea sections, we decided to investigate the possible effects of adenine upon the growth of the lateral buds of etiolated pea epicotyls.

Using the technique described under *Methods*, we found that buds develop very slowly if the epicotyls are supplied only with 2% sucrose and phosphate buffer. However, the addition of small quantities of adenine or related compounds to the solution will greatly enhance the growth of buds in the light (Table III). Although adenine did seem to

TABLE III

Effect of Adenine and Related Compounds on Growth of Buds of Node No. 2 of Etiolated Pea Epicotyls

Epicotyls placed erect in 10 cc. of test solution. Illumination = 500 f.c. fluorescent light. Harvested after 7 days.

Experiment no.	Test solution	Fresh wt. buds/ 10 plants (mg.)	Length of buds (mm.)
P-38	Control (2% sucrose+pH 6.1 phosphate buffer)	58	
	Control+10 γ/cc. IAA	18	
	Control+10 γ/cc. adenine	136	
	Control+10 γ/cc. adenine+10 γ/cc. IAA	28	
P-66	Control (as in P-38)	70.3	5.2±0.40[a]
	Control+10 γ/cc. adenine	99.0	6.8±0.64
	Control+10 γ/cc. adenosine	103.4	7.1±0.46
	Control+10 γ/cc. adenylic acid	116.0	8.1±0.85
P-67	Control (as in P-38)	19.6	3.1±0.04
	Control+10 γ/cc. adenine	37.8	4.8±0.05
P-78	Control (as in P-38)	14.4	
	Control+50 γ/cc. adenylic acid	143.2	

[a] Standard error.

produce a small increase in the growth of such buds in the dark, the overall growth of all buds was poor in the dark. This indicates that some factor other than adenine, essential for the growth of buds, is produced only in the light.

From the data of Expt. P-38, it may be seen that the growth stimulation produced by adenine is almost completely reversed by 10 γ/cc. IAA, which is known to inhibit the development of lateral buds (12). It was found in subsequent experiments that very small concentrations of IAA (down to 10 γ/l.) were effective in producing this inhibition, and that adenine could not appreciably reverse such an inhibition. Obviously, then, the rate of growth of the controls and of the adenine-treated buds would be affected by the endogenous auxin levels of the tissues. The variation in bud growth from one experiment to another may, therefore, be attributed to differences in auxin content of the experimental plants.

Whereas the data of Table III show a definite stimulation of bud growth by adenine, the fact that the experiments were not run aseptically might cast some doubt on the *direct* participation of adenine in the bud-growth phenomenon. It is not practicable to study the growth of node No. 2 under aseptic conditions, but the terminal bud of young sterile seedlings may be conveniently excised and cultured. Therefore, sterile cultures of such buds were made, and the effect of adenine on their growth studied.

The peas were soaked in tap water for 30 min., sterilized in 1:10 Purex for 5 min., then implanted in sterile Petri dishes containing a shallow layer of 1% agar. The plates were incubated in the dark at 27°C., and the terminal buds were excised 4 days later. Eight buds were floated on the surface of 10 cc. of nutrient solution in a 50 cc. Erlenmeyer flask and were allowed to grow in the light for 31 days. They were then harvested, and their fresh weight determined. The control nutrient solution contained 2% sucrose, 236 mg./l. $Ca(NO_3)_2 \cdot 4H_2O$, 36 mg./l. $MgSO_4 \cdot 7H_2O$, 81 mg./l. KNO_3, 65 mg./l. KCl, 20 mg./l. KH_2PO_4, and 1.5 mg./l. Fe tartrate. The results, shown in Table IV, clearly demonstrate that adenine caused a great increase in growth of these terminal leaf buds.

TABLE IV

The Effect of Adenine on the Growth of Apical Buds of Etiolated Peas in Pure Culture

Eight buds floated on 25 cc. of nutrient medium in the light, and harvested after 31 days of incubation at 27°C.

Solution	Fresh weight of buds (mg.)
Control (2% sucrose + minerals)	173.4
Control + 10 γ/cc. adenine	538.6

C. Experiments on Root Initiation

When etiolated pea epicotyls are exposed to IAA, many root primordia are initiated at the lower end of the epicotyl. This phenomenon is the basis of a quantitative root initiation procedure (13). During the experiments on bud growth which were carried out in the dark, we noticed that the number and length of the roots produced on these epicotyls were greater in the adenine-treated plants than they were in the controls. Experiments were, therefore, set up to measure this effect quantitatively.

Ten cm. long epicotyl sections were inverted for 24 hr. in 10 cc. of the test solution, and were then reinverted into 2% sucrose + pH 6.1 phosphate buffer and permitted to stand for an additional 6 days in the experimental dark chamber. Light was excluded because of its inhibitory effect upon root initiation (1,3,14). At the end of the 7-day experimental period, the number of macroscopically visible roots on each epicotyl was counted. The results, shown in Table V, indicate that adenine increases the number of roots formed in the presence of 10 γ/cc. IAA.

TABLE V

The Effect of Adenine and Adenosine on Root Initiation in Etiolated Peas

Twelve 10 cm. epicotyl sections containing node No. 2 inverted for 24 hr. in test solution, then reinverted, put into 2% sucrose + buffer. Experiment conducted in dark room at 25°C.

Expt. no.	Test solution	Number of roots per epicotyl
P-48	Control (2% sucrose + pH 6.1 phosphate buffer + 10 γ/cc. IAA)	4.7±0.94[a]
	Control + 10 γ/cc. adenine	8.1±1.14
P-213	Control (as in P-48)	3.5±1.11
	Control + 10 γ/cc. adenosine	5.5±1.16
P-227	Control (as in P-48)	4.3±0.73
	Control + 10 γ/cc. adenosine	6.8±0.86

[a] Standard error.

DISCUSSION

The data presented in this paper show that adenine may stimulate the growth of 3 different organs of the etiolated pea: epicotyl, roots and leaf buds. It may, therefore, be considered that adenine synthesis proceeds too slowly in the etiolated pea plant to accommodate the growth requirements for this compound.

Went (15) on the basis of various excision experiments with etiolated peas, found that, in addition to auxin, other factors are required for normal growth and development. He postulated the existence of three new hormones: *caulocaline*, formed in the roots, essential for stem elongation and bud growth; *rhizocaline*, stored in the cotyledon, essential for root initiation; *phyllocaline*, stored in the cotyledon, essential for leaf growth. Subsequent investigators, (*e. g.*, 16) have reported that effects attributed to these specific *calines* may be duplicated by known substances, such as arginine, *etc.* The results of the present investigation indicate that adenine may produce effects in the etiolated pea plant ascribed to all three of the postulated calines. It would, therefore, seem justifiable to assign to adenine the role of a *general* growth factor in etiolated peas, which is perhaps identical with one or all of the *calines*. Although it is quite clear that adenine may be generally stimulatory in the etiolated pea plant, certain other compounds may also produce stimulatory effects on bud growth, root growth, and section growth. Therefore, adenine may not be the only substance actually functioning as the *caline*.

It is interesting to note that the effects of adenine on epicotyl section growth and upon root initiation are manifested only in the presence of IAA, whereas the stimulation of bud growth is antagonized by IAA. Similar findings have recently been reported by Skoog and Tsui (11).

SUMMARY

1. Adenine stimulates the growth of epicotyl sections, leaf buds and roots of etiolated peas.

2. The thermal inactivation of the growth of excised pea epicotyl sections may be largely prevented by the addition of adenine to the medium.

3. It is suggested that the etiolated pea plant is incapable of synthesizing enough adenine for its growth needs, and that the various *calines* hypothesized by Went may be identical with adenine.

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The Metabolism of the Heart in Relation to Drug Action. I. The Endogenous Aerobic Metabolism of Rat Heart Slices [1]

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INTRODUCTION

The primary objective in the present investigation of the action of drugs on the heart is the elucidation of the reactions between the drugs and metabolic systems in the myocardium, as it has been shown that many drugs which alter the functional activity of the heart act directly on enzyme systems. We have first investigated various aspects of cardiac metabolism in order to establish a basis for subsequent work with drugs.

It is preferable in work of this type to limit the investigations to one species of animal to correlate data from various lines of approach. We have chosen the rat, and have made some preliminary studies of the aerobic metabolism of rat heart slices, in the absence of added substrate.

METHODS AND MATERIALS

The oxygen consumption of rat heart slices was determined in a standard Warburg manometer apparatus. In all experiments the temperature was 37°C. and, unless stated otherwise, the gas mixture used was air. The slices were suspended in 3 cc. of a modified Krebs-Ringer-phosphate medium (1). Two modifications were made in the original medium: the pH was adjusted to 7.4 and the calcium concentration was reduced by one-half. The medium prepared as described by Krebs and Henseleit possessed a pH of approximately 7.1; to approach the pH of the blood more closely it was felt that the pH should be adjusted to 7.4. However, when this was done, some of the calcium precipitated as a phosphate complex. When the calcium concentration was reduced to one-half, no precipitate formed. It is noteworthy that this reduced calcium concentration corresponds more closely to the free ionic calcium concentration in the blood.

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Normal young adult rats of 150–250 g. were used. The animal was decapitated, the heart excised, and immediately placed on ice-cold filter paper moistened with medium. Longitudinal slices of ventricle were made with a Stadie slicer (2); a slice thickness of 0.3 mm. was used throughout, unless otherwise stated. The slices were cut into halves or thirds because of the tendency of large slices to fold over or roll up on themselves, possibly limiting the diffusion of oxygen. The average amount of heart tissue placed in each flask was 55–65 mg. wet weight. Several pieces of tissue from various regions of the heart were selected for each vessel to minimize variations due to differences in metabolism between these regions. The total time between removal of the heart and placing the vessels in the bath was about 5 min. Equilibration time in the bath was uniformly 15 min. All Q_{O_2} values are expressed on a dry weight basis. The results are plotted showing the rate of oxygen uptake during each time interval, rather than plotting the accumulated oxygen uptake; in this way changes in the respiratory rate are more clearly seen.

EXPERIMENTAL

Rate of Endogenous Respiration

The initial rate of respiration of the heart slices in the absence of added substrate, as averaged over the first 20 min. after equilibration, corresponded to a Q_{O_2} of 3.53; this is the mean value of the oxygen uptake from 341 determinations. The average deviation in the Q_{O_2} was 0.125, and the probable error of the mean was ±0.007. The distribution of these initial Q_{O_2} values is shown in Fig. 1, from which it can be seen that there is appreciable variation among individual rats; variation between vessels containing heart slices from a single rat is much less. These variations could not be correlated with age, weight or sex of the rats. Because of this variation, in comparing the respiration of heart slices under various conditions it is important to make these comparisons within an experiment on a single rat heart, and to use the appropriate number of rats to obtain a quantitative result.

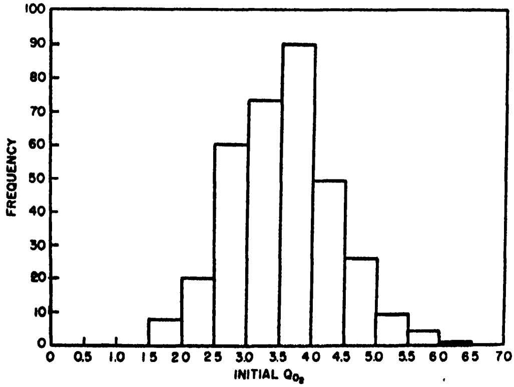

FIG. 1. Distribution of initial Q_{O_2} values in 341 experiments on rat heart slices.

Course of Endogenous Respiration

The respiration of rat heart slices in the absence of added substrate fell progressively with time in a manner shown by the curve in Fig. 2, which was obtained by averaging the results of 66 control curves. During the first 3 hr., the rate dropped to approximately 60% of its original value. The addition of nicotinamide (0.002–0.01 *M*) to the medium had no effect on the rate of falling off of the respiration.

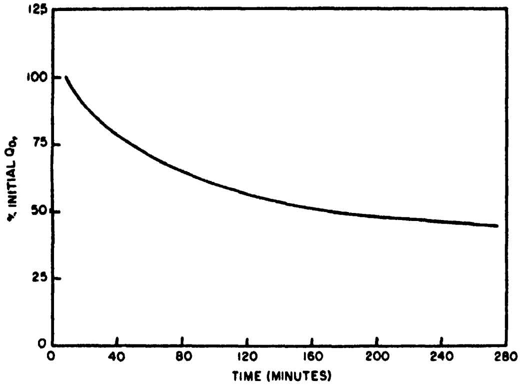

FIG. 2. Course of the endogenous respiration of rat heart slices obtained by averaging 66 control curves.

Slice Thickness

In work on tissue slices it is important to demonstrate that the rate of diffusion of oxygen into the slice does not limit the respiratory rate. In air, the maximum permissible thickness of the tissue slice, according to the formula of Warburg (3), was calculated to be 0.42 mm., using the average initial endogenous Q_{O_2}. In several experiments it was shown that there was no significant difference in oxygen uptake between slices 0.3 mm. and 0.4 mm. in thickness. However, when the thickness was increased to 0.5 mm. the respiration dropped off about 30%, indicating that at this thickness oxygen diffusion was probably a limiting factor. Since the respiration of heart slices is often higher than the average value used in the above calculation, it was believed that the use of slices 0.3 mm. thick would provide a margin of safety.

Effect of Pure Oxygen

When the gas phase in the vessels was pure oxygen, the rat heart slices respired at a rate 125% higher than when the gas phase was air; the averages of 3 experiments gave $Q_{O_2}^{air}$ as 2.21 and $Q_{O_2}^{O_2}$ as 4.97 over the first hour (the respiration was somewhat lower than in the usual experiments because of the 15 min. gassing which delayed the starting of readings). In pure oxygen the respiration was better maintained for the first 2 hr. but afterwards decreased more rapidly than in air, as is shown in Fig. 3.

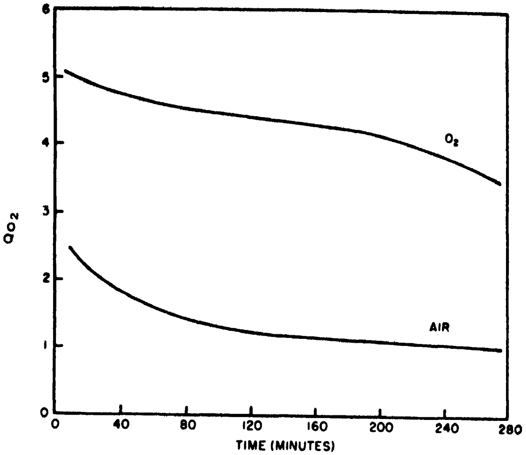

Fig. 3. Course of endogenous respiration of rat heart slices in air and in pure oxygen.

Effect of Anoxia

Short periods of myocardial anoxia decreased the rate of respiration markedly. Table I gives the results of experiments in which the heart was allowed to remain in the animal after decapitation for 5 min. and 15 min. before it was sliced; it is evident that the metabolic damage progresses rapidly during periods of anoxia. These results, in general, confirm the work of Bernheim and Bernheim (4) and indicate the importance of rapid removal of the heart from the animal and immediate slicing.

Depression of respiration was also observed after the heart slices were exposed to anoxic conditions in the Warburg vessels. Normal respiration readings were made for 40 min. in 6 vessels. Two vessels were then gassed with nitrogen for 20 min. and 2 vessels for 60 min.; the nitrogen was removed by gassing with air for 5 min. and the vessels were allowed to equilibrate. Two control vessels were gassed only with air to make them comparable with the other vessels except for the periods of anoxia. The results are represented in Fig. 4. When the respiration rates for 1 hr. periods following anoxia are compared, we find that 20 min. anoxia reduced the oxygen uptake to 40%

TABLE I

Effect of Myocardial Anoxia on Respiration

Period of anoxia	No. of expts.	Initial Q_{O_2} (0–10′)	Per cent of control
Control	12	−3.45	100
5 min.	6	−2.20	64
15 min.	6	−0.82	24

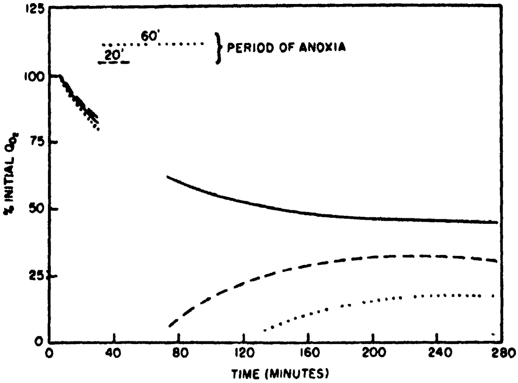

FIG. 4. Effect of anoxia on the respiration of rat heart slices. Full line: control. Dashed line: anoxic period of 20 min. Dotted line: anoxic period of 60 min.

of the control slices, and that 60 min. anoxia gave a reduction to 23% of the control; the initial respiratory depression was even greater and from the curves it can be seen that it must have been almost complete immediately following the period of anoxia. It is interesting to observe that partial recovery from the anoxic depression occurred.

Effect of pH

The respiration of heart slices was found to be more sensitive to alkaline than to acid conditions as indicated by the results given in Table II. The determinations above pH 7.4 were made in the absence of calcium from the medium (to avoid precipitation of calcium phosphates) and compared with controls at pH 7.4 without calcium. Further work showed that a decrease of the pH of the medium as far as 6.0 induced no significant change in the oxygen consumption. It is obvious that the slight decrease in pH of the medium during the course of an experiment would have very little effect upon the oxygen uptake.

TABLE II

Effect of pH of Medium on Respiration

pH	No. of expts.	Average Q_{O_2} over 4 hr.		Per cent of Q_{O_2} at pH 7.4
		Calcium	No calcium[a]	
7.0	2	−2.54		97
7.2	2	−2.57		98
7.4	4	−2.62	−2.36	100
7.6	2		−2.06	87
7.8	2		−1.53	65

[a] See text.

Effect of Changes in Calcium and Potassium Concentration

Aside from the intrinsic interest in the actions of calcium and potassium on the heart, there is occasionally the problem of investigating the action of some substance that removes calcium from the medium (*e.g.*, citrate or pyrophosphate), in which case calcium must be omitted; it is of importance to know what changes in respiration are brought about by this deletion. Berheim and Bernheim (4) stated that, in the rat heart, there is little or no effect of removal of either potassium, calcium or magnesium singly from the medium, but that respiration is markedly depressed if all three are omitted. Levy and Schwob (5) reported results on the rat heart that are essentially in agreement with the above; they found that either potassium or calcium in the medium allowed a normal oxygen consumption, and that magnesium concentration was of little importance until its concentration reached 3 times the normal level, when it produced about 20% inhibition. Our results are recorded in Table III from which it is evident that lack of calcium in the medium causes a progressive depression of the respiration, the effect being small initially but amounting after 4 hr. to approximately a 50% inhibition.

TABLE III

Effect of Calcium and Potassium Concentration on Respiration

Calcium m*M*	Potassium m*M*	No. of expts.	Q_{O_2}			Per cent of control		
			0-10′	230 240′	0-60′	0-10′	230-240′	0-60′
1.37[a]	6.47[a]	13	−3.76	−1.52	−3.24	100	100	100
0	6.47	13	−3.54	−0.78	−2.94	94	51	91
1.37	6.47	8	−4.49	−1.07	−3.80	100	100	100
1.37	1.28	8	−3.64	−0.85	−2.88	81	80	76

[a] Concentrations in standard medium.

The effect of reducing the potassium concentration in the medium is also given in Table III. The depression of respiration here is not progressive with time and the inhibition after 4 hr. is almost identical with the initial inhibition. The effect of increasing the potassium concentration in the medium 5-fold and 25-fold is negligible.

DISCUSSION

Investigations of the metabolism of tissue slices require a consideration of the degree of correspondence between this metabolism and that of the intact tissue in the animal. The oxygen consumption of intact or perfused isolated rat heart is not known, but the Q_{O_2} of mammalian (cat, rabbit and dog) heart in heart-lung type preparations lies between 8 and 30 mm.^{3}/mg./hr. These values were obtained on beating hearts in the presence of substrate. Since the ventricular slices in the present work were not rhythmically beating and were respiring in the absence of substrate, it is not surprising that the oxygen uptake was less than in the experiments quoted above.

The metabolic depression resulting from short periods of anoxia is apparently not due to destruction of coenzyme I or cocarboxylase. Bernheim and Bernheim (4) found that heart tissue that had been made anoxic would oxidize pyruvate and lactate as readily as normal tissue, indicating that the enzymes and coenzymes involved are still intact. Furthermore, Govier (6) has shown that in dog heart the coenzyme I decreased only 25.9% and the cocarboxylase only 13.7% during 1 hr. of anoxia.

SUMMARY

1. To establish a foundation for future work on the actions of drugs on the heart, the endogenous aerobic metabolism of rat heart has been investigated by determining the oxygen uptake of heart slices under various conditions.

2. The effect of the following factors upon respiration has been determined: thickness of the slice, oxygen tension, myocardial anoxia, pH of the medium, and the concentrations of calcium and potassium in the medium.

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The Metabolism of the Heart in Relation to Drug Action. II. The Utilization of Substrates by Rat Heart Slices[1]

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INTRODUCTION

Investigations of cardiac metabolism are always of interest not only in themselves, but also because of the possibility of utilizing substrates and enzyme factors, alone or in combination with drugs, in the treatment of heart disease. It is of importance to know which substrates the heart can oxidize and from which it can derive energy for muscular contraction. We have, therefore, investigated the effects of a number of substrates on the respiration of rat heart slices in connection with work on the metabolic effects of various cardiovascular drugs.

METHODS AND MATERIALS

The oxygen consumption of rat heart slices was determined as described in the previous paper (1). Substrates were dissolved in the modified Krebs-Ringer-phosphate medium and, in every case, adjusted to pH 7.4. These substrates were either present in the vessel initially or were added from the side arms at various times; in the latter case they were made up to 10 times the final concentration desired.

Glucose, hexose diphosphate (Schwarz), fumaric acid, *l*-malic acid, L-(+)-glutamic acid, *n*-caprylic acid, sodium citrate and sodium acetate were obtained from commercial sources. Glucose-1-phosphate was prepared by the action of potato phosphorylase extract upon soluble starch, with subsequent purification according to the method of Sumner and Somers (2). It was approximately 70% pure. Sodium succinate was purified by the method of Potter and Schneider (3). Lactic acid was diluted 1:9, boiled for 5 hr. to break down the anhydride, and neutralized. Pyruvic acid was redistilled and the fraction boiling at 60–70°C./20–25 mm. was collected; the sodium salt was prepared by the method of Robertson (4). Oxalacetic acid (m.p. 143–7°C.) was synthesized from diethyloxalacetate by the method of Krampitz and Werkman

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(5). α-Ketoglutaric acid (m.p. 110°C.) was synthesized by the method of Friedman and Kosower (6). Sodium β-hydroxybutyrate was prepared from the Ca-Zn double salt, which was isolated from the urine of fasted rats fed sodium butyrate after the method of Blunden (7).

The results were calculated and plotted in a manner similar to that of the previous paper (1).

EXPERIMENTAL

Effect of Substrates on Respiration

To determine the effect of a substrate on the oxygen uptake of rat heart slices, six 10-min. control readings were made during the first hour after equilibration, the respiration by this time having achieved a fairly steady rate of decrease; the substrates were then added in various concentrations from the side arms. The change in respiration due to the substrate was recorded in terms of per cent of the last control reading and is corrected for the amount of change during the 10-min. period, following the addition of the substrate, in the control vessel to which no substrate had been added. Examples of the curves obtained from such experiments are given in Fig. 1 for 4 different substrates; from these curves we see that, in this particular experiment, the percentage change in respiration due to malate was 25%, to pyruvate 35%, to citrate 68%, and to succinate 238%.

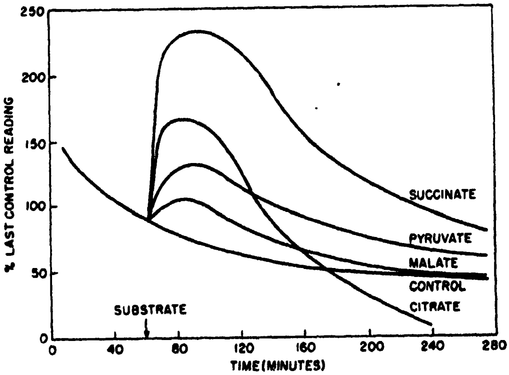

FIG. 1. Typical effects of four substrates on the respiration of rat heart slices. Substrate concentrations all 0.01 *M*.

The effects of the substrates were determined at concentrations from 0.001 *M* to 0.05 *M* in most cases and the average results obtained are given in Table I, the figures in parentheses showing the number of determinations made.

The initial effect in the first 10 min. period following the addition of the substrate is given for all concentrations, and, in addition, the effect over the first hour is also given for concentrations of 0.01 *M* and 0.02 *M*. The approximate increases in Q_{O_2} due to the addition of substrate may be calculated by multiplying the percentage change by 2.90, the average Q_{O_2} of the last control reading before the substrate was added, and dividing by 100.

TABLE I

Effects of Substrates on the Oxygen Uptake by Rat Heart Slices

Substrate	Per cent increase in Q_{O_2} at varying substrate concentrations							
	0–10′ effects						0–60′ effects	
	0.001 *M*	0.002 *M*	0.005 *M*	0.01 *M*	0 02 *M*	0 05 *M*	0.01 *M*	0.02 *M*
Succinate	73 (1)	108 (1)	343 (8)	356 (1)	348 (6)	286 (1)	320 (1)	342 (6)
Citrate	57 (1)	—	63 (1)	56 (1)	87 (1)	36 (1)	42 (1)	50 (1)
Oxalacetate	26 (2)	—	39 (1)	103 (5)	52 (2)	—	74 (5)	41 (2)
Pyruvate	13 (2)	26 (4)	13 (2)	34 (8)	21 (2)	10 (1)	29 (8)	24 (2)
Fumarate	—	13 (2)	27 (1)	24 (4)	14 (2)	16 (1)	17 (4)	13 (2)
Glutamate	—	18 (1)	11 (2)	16 (2)	23 (2)	18 (2)	11 (2)	15 (2)
Lactate	—	18 (1)	24 (4)	18 (2)	13 (1)	—	17 (2)	17 (1)
Malate	9 (2)	13 (2)	17 (2)	22 (3)	29 (2)	24 (2)	21 (3)	22 (2)
Acetate	—	15 (5)	—	—	5 (2)	—	—	8 (2)
β-Hydroxybutyrate	—	4 (1)	0 (1)	5 (1)	11 (1)	0 (1)	8 (1)	15 (1)
Caprylate	—	6 (1)	8 (1)	−20 (1)	−68 (1)	−83 (1)	−28 (1)	−80 (1)
α-Ketoglutarate	—	−2 (1)	−1 (1)	−2 (1)	−7 (1)	−4 (1)	−4 (1)	−10 (1)
Glucose	—	0 (1)	0 (1)	0 (2)	0 (7)	0 (1)	0 (1)	4 (7)
Glucose-1-phosphate	—	—	—	0 (3)	—	—	0 (3)	—
Hexose diphosphate	—	—	—	—	0 (6)	—	—	−22 (6)

The figures in parentheses give the number of determinations made.

It was shown in the previous paper (1) that there was quite wide variation in the endogenous respiration from one animal to another; likewise, there is marked variation in the respiratory response of the heart slices to the addition of substrates. In the case of pyruvate (0.01 *M*) the individual increases in respiration were 14, 16, 28, 39, 40, 42, 43, and 50%, representing an average deviation from the mean of 11% and a probable error of the mean of 4%. Similarily with oxalacetate (0.01 *M*) the individual increases were 73, 92, 98, 118, and 136%, the average deviation being 19% and the probable error of the mean 8.5%. The concentration of the substrate that gave the maximum increase was usually between 0.01 *M* and 0.02 *M*. At higher concentrations there was frequently inhibition, which was particularly marked later in the experiment, a fact which in many instances indicates damage to the tissue by the substrate or the products formed by the breakdown of the substrate. This inhibition was noted especially with citrate and caprylate. There was no evidence of alteration of the tissue or its metabolism by osmotic forces brought into play by the addition of the substrate, *e.g.*, glucose, in a concentration of 0.1 *M* (1.8%), did not produce any increase nor decrease in the rate of falling off of the oxygen uptake.

The results given in Table I indicate that the substrates can be divided into four general classes with regard to their effects on the respiration of rat heart slices. This classification is based upon the immediate effects (0–10 min. readings) of adding the substrates after 1 hr. of control readings.

Class I (increase respiration 200–400%)—succinate.
Class II (increase respiration 50–100%)—citrate and oxalacetate.
Class III (increase respiration 5–35%)—pyruvate, fumarate, glutamate, lactate, malate, β-hydroxybutyrate, caprylate, and acetate.
Class IV (fail to increase respiration)—glucose, glucose-1-phosphate, hexose diphosphate, and α-ketoglutarate.

Utilization of Glucose

It is clear from Table I that glucose, at various concentration, had no significant effect on oxygen uptake during the first hour following its addition to respiring heart slices. However, when 0.02 *M* glucose was present in the medium from the start of the experiment, the rate of respiration reached a steady state at 1–2 hr., instead of continuing to decrease like the controls, and at 2–4 hr., the rate increased slowly; in some instances it became greater than the rate which immediately followed the 15 min. equilibration period. In Fig. 2, the curves illustrating these effects represent the average of 30 experiments on 8 hearts.

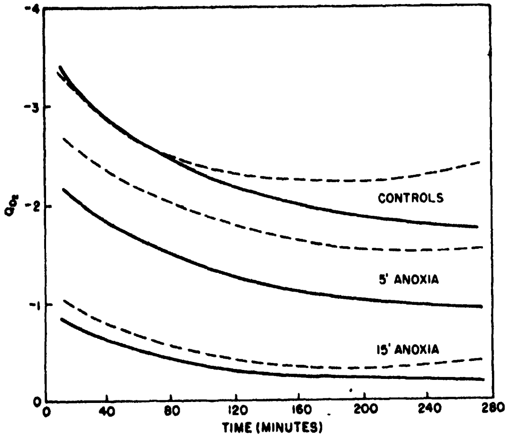

FIG. 2. Effect of periods of anoxia on the endogenous respiration of rat heart slices and the action of glucose on the normal and anoxic tissue. Solid lines: endogenous respiration. Dashed lines: in the presence of 0.02 *M* glucose.

It has been shown that short period of anoxia depressed the respiration of the heart (1). The effect of glucose was investigated on the oxygen uptake of slices prepared from hearts which had been made anoxic by allowing them to remain in the animal for 5 to 15 min. after decapitation. The action of glucose on the respiration of slices from such anoxic hearts was more rapid and marked than on those from normal hearts, as shown in Fig. 2. Hearts that were made anoxic for 5 min. gave a Q_{O_2} of 1.94 during the first hour in the absence of substrate and 2.70 in the presence of glucose, an increase of 39% due to glucose. Hearts that were made anoxic for 15 min. gave an endogenous Q_{O_2} of 0.54 compared to 0.78 in the presence of glucose, an increase of 44%. Normal hearts showed no effect from glucose during the first hour of the experiment. Anoxia thus altered the cardiac metabolism in such a manner that the utilization of exogenous glucose was facilitated.

DISCUSSION

It has been clearly shown that the isolated mammalian heart when perfused, or in the heart-lung preparation, can utilize glucose for the energy of contraction (8, 9, 10 and others). We have found that glucose is readily utilized by the perfused rat heart; when the heart has been made hypodynamic by perfusion without substrate, glucose rapidly restored the heart to a normal rate and amplitude (11). Wollenberger (16) has shown that glucose does not raise the respiration of guinea pig heart slices. We found no immediate effect from the addition of glucose at concentrations from 0.002 *M* to 0.1 *M*, but there was a delayed increase in respiratory rate with 0.02 *M* glucose 1–3 hr. after the start of the experiment. In hearts damaged by periods of anoxia, the effect of glucose is more rapid and results in a 40–45% stimulation of the oxygen uptake. A possible explanation is that anoxia reduces the ability of the heart to utilize its endogenous substrate, and that glucose is then able to be utilized as it is when gradual depletion of the endogenous substrate occurs. Another explanation might be that anoxia alters the metabolic pattern in such a manner that glucose can be broken down by alternate pathways that are normally insignificant.

Utilization of lactate has been shown in the perfused dog heart and the dog heart-lung preparation (9, 17, 18). Conflicting results have been obtained with rat heart slices (12, 13, 15). We have found a consistent 10–20% stimulation of oxygen uptake by concentrations of lactate between 0.002 *M* and 0.02 *M*.

There is unanimity of opinion that the mammalian heart can utilize pyruvate. With regard to the perfused dog heart, Braun-Menendez, Chute, and Gregory (19) reported utilization at various concentrations, and we have shown that pyruvate will restore the contractions of the hypodynamic perfused rat heart (11). Various workers have demonstrated the utilization of pyruvate by heart slices of rat (12, 14, 15, 20), and guinea pig (16). We have found a 10–35% stimulation of the respiration of rat heart slices with concentrations from 0.0002 *M* to 0.05 *M*. In experiments to be described in a later report (21) malonate was observed to inhibit the utilization of pyruvate by heart slices; it is probable, then, that the latter substrate is being metabolized *via* the Krebs cycle.

Rat heart slices are able to oxidize succinate with great rapidity; we observed increases in oxygen uptake of 75–400% with succinate in concentrations of 0.001 *M* to 0.05 *M*. In fact, succinate is oxidized so readily that, at the lower concentrations, all is oxidized within 2–3 hr., as calculated from the increased oxygen consumption. Other workers have reported increased oxygen uptake by heart slices of rat (12, 15) and guinea pig (16) following the addition of succinate.

In our experiments, fumarate, glutamate, acetate, citrate, oxalacetate, malate, and β-hydroxybutyrate also increased the oxygen uptake. Bernheim and Bernheim (12), however, reported that rat heart slices did not oxidize fumarate, glutamate, or acetate, and Wollenberger (16) that the Q_{O_2} of guinea pig heart slices was not increased by acetate.

SUMMARY

1. Fifteen substrates were tested at various concentrations for their effects on the respiration of rat heart slices. These substrates were classified on the basis of their *immediate* effect on respiration as follows: (I) increase respiration 200–400%—succinate; (II) increase respiration 50–100%—citrate and oxalacetate; (III) increase respiration 5–35%—pyruvate, fumarate, glutamate, lactate, malate, β-hydroxybutyrate, caprylate, and acetate; (IV) fail to increase respiration—glucose, glucose-1-phosphate, hexose diphosphate, and α-ketoglutarate.

2. Glucose had no immediate effect on the oxygen uptake of slices from normal hearts, but caused a delayed stimulation of respiration several hours after the beginning of the experiment. Glucose also increased the oxygen uptake of slices from hearts that had been made anoxic for 5 and 15 min.

3. The members of the tricarboxylic acid cycle increased the oxygen uptake to varying degrees, but none could fully maintain respiration; that is, they did not, after their initial stimulation of oxygen uptake, alter markedly the rate of decrease of respiration.

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The Metabolism of the Heart in Relation to Drug Action. III. The Action of Metabolic Inhibitors on Rat Heart Slice Respiration [1]

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INTRODUCTION

It is likely that, in the analysis of the actions of drugs upon the cellular metabolism of the heart, it will be advantageous to be able to inhibit certain enzyme systems or separate one phase of metabolism from another; this can be accomplished to a limited degree by the judicious use of certain enzyme inhibitors that are reasonably specific. For this reason we have investigated the actions of several of these inhibitors on the oxygen uptake of rat heart slices.

METHODS AND MATERIALS

The oxygen consumption of rat heart slices was determined as described in a previous paper (1). Substrates and inhibitors were dissolved in modified Krebs-Ringer-phosphate medium and, in every case, adjusted to pH 7.4. These substances were either present initially or were added from the side arms at various times; in the latter case the solutions were made up to 10 times the final concentration desired. The substrates used were described in the previous paper (2), and the inhibitors obtained from commercial sources. The sodium salts of acids were used. The results were calculated and plotted in a manner similar to that of a previous paper (1).

In the experiments with cyanide the appropriate mixtures of KOH and KCN solutions, corresponding to the pH of the medium and the concentration of cyanide, were added to the center well to eliminate passage of HCN between the center well fluid and the medium.

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EXPERIMENTAL

Malonate

When malonate was present in the medium from the beginning of the experiment, there was an inhibition of the initial rate of respiration of the rat heart slices (average of the rate over the first 10 min. after equilibration) as shown in Table I. If malonate was added 1 hr. after the start of the experiment, there was also an inhibition but the effect was less marked (average of the rate over the period from 65 to 85 min.). When the oxygen uptake of the heart slices was increased by 0.02 *M* succinate, this increased respiration was markedly inhibited by malonate after correction was made for the endogenous respiration that is proceeding simultaneously with the oxidation of succinate. These results with malonate are summarized in Fig. 1. The inhibition of the endogenous respiration, both when malonate is present initially and when it is added after 1 hr., was proportional to the log of the malonate concentration.

TABLE I

Initial Malonate Inhibition of Respiration

Malonate	No. of expts.	Q_{O_2}	Inhibition
M		*0–10 min.*	*per cent*
0	19	−3.81	—
0.002	2	−3.39	11
0.005	8	−3.02	21
0.010	6	−2.58	32
0.020	11	−2.28	42
0.050	3	−1.46	62
0.100	1	−1.14	70

In Fig. 1 it can be seen that, when malonate was present in the minimal concentration for the complete inhibition of succinate oxidation, there was approximately 50% inhibition of the initial endogenous respiration and 25% inhibition of the endogenous respiration when malonate was added 1 hr. later. It seems likely, therefore, that only part of the endogenous respiration of the rat heart involves the succinate oxidation system, and that the fraction of the respiration utilizing this system varies with the condition of the heart slices. Inhibition of respiration over 50% with higher concentration of malonate was probably due to inhibition of enzyme systems other than that oxidizing succinate; it has been shown that malonate in this concentration range will inhibit dehydrogenases involved in the oxidation of other dicarboxylic acids.

When malonate was present from the beginning of the experiment, the initial respiration was inhibited as described above, but the subsequent course of the respiration demonstrated an unexpected phenomenon. The oxygen uptake rose during the first 30–60 min. to reach a peak and then fell off more slowly than the control. This occurrence of a hump in the respiration curve was seen with concentrations of malonate between 0.002 *M* and 0.05 *M*. Fig. 2 shows the average results obtained in such

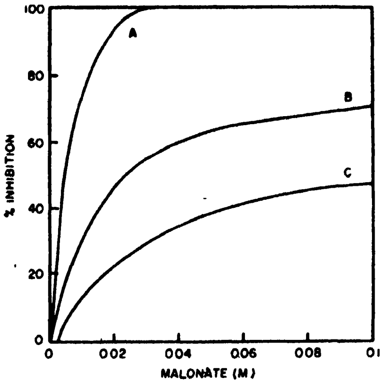

Fig. 1. Inhibition of respiration of rat heart slices by different concentrations of malonate. Curve A: inhibition of succinate (0.02 *M*) oxidation. Curve B: inhibition of initial (0–10 min.) endogenous respiration. Curve C: inhibition of 65–85 min. endogenous respiration; malonate added at 60 min. (see text).

experiments; the curves for the concentrations 0.005 *M*, 0.01 *M* and 0.02 *M* are each an average of 6 experiments, the curve for 0.05 *M* is the average of 3 experiments, and the control curve is the average of all the control curves in these experiments. The respiration in the presence of 0.005–0.02 *M* malonate almost invariably rose above the respiration of the controls and remained higher for at least 3 hr. Malonate, therefore, can maintain respiration higher that the controls, although its initial action is inhibition. Turner and Hanly (3) have also observed stimulation of oxygen-

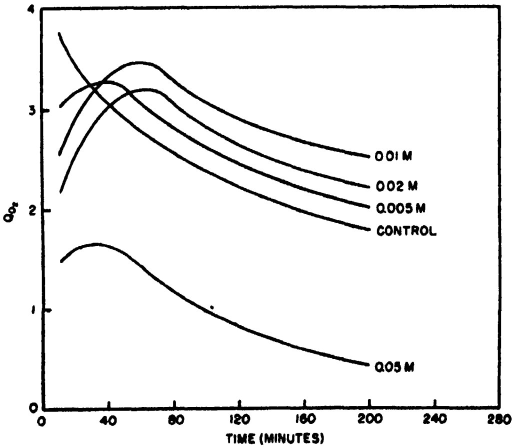

Fig. 2. Effect of various concentrations of malonate, present at the beginning of the experiment, on the respiration of rat heart slices.

uptake with malonate on slices of carrot root. We did not observe this phenomenon with any of the other inhibitors used.

The rise in respiration and the better maintenance of respiration induced by malonate were inhibited by 0.005 *M* fluoride as shown in Fig. 3.

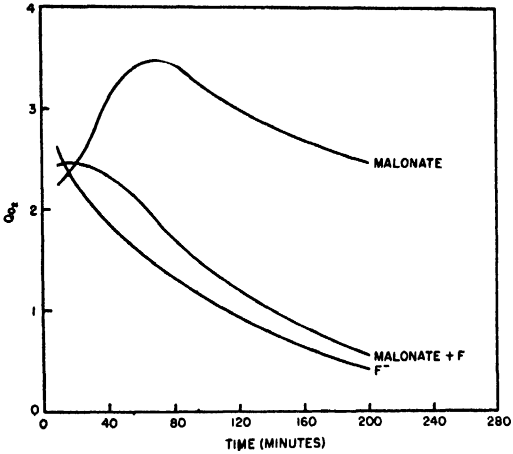

Fig. 3. Action of fluoride (0.005 *M*) on the malonate (0.01 *M*) effect on respiration of rat heart slices.

In several experiments comparing malonate with pyrophosphate, in which calcium was omitted from the medium in order to prevent precipitation of the pyrophosphate, it was observed that malonate did not produce the typical rise in respiration as described above. Consequently, we conducted experiments to determine the effect of calcium on the action of malonate and found that calcium was necessary for the typical response. In the absence of calcium, malonate (0.01 *M*) increased the Q_{O_2} from 1.18 to 1.32 (increase of 7%); in the presence of calcium, the Q_{O_2} was increased from 1.70 to 2.78 (increase of 64%). Malonate was present throughout the experiment in half the vessels and these were compared with control vessels containing no malonate; the Q_{O_2} values were averaged over the period from 70 to 200 min., thus avoiding the first hour during which the malonate was exerting its initial depression. There was a slight stimulation of respiration by malonate in calcium-free medium, but this was insignificant compared to the effect in the presence of calcium.

The increase in respiration brought about by the addition of fumarate (0.01 *M*) was not altered by malonate (0.02 *M*), as would be expected on the basis of the action of malonate on succinic dehydrogenase. The effect of pyruvate (0.01 *M*) on the respiration was partially inhibited by malonate (0.02 *M*): in the control experiments pyruvate gave increases in respiration of 39%, 40% and 42% (average of 40.3%), but in the presence of malonate the increases were 10%, 13% and 14% (average of 12.3%), corresponding to a 70% depression of the pyruvate effect. This also would be expected if pyruvate were being utilized through the tricarboxylic acid cycle, since malonate inhibits the functioning of this cycle.

Iodoacetate

The endogenous respiration of rat heart slices was found to be inhibited strongly by iodoacetate. The results are summarized in Table III where its inhibitory potency may be compared to that of other inhibitors. Not only was the initial oxygen uptake inhibited, but the respiration in the presence of iodoacetate fell off more rapidly than the control; that is, the inhibition became progressively greater with time. This progressive action of iodoacetate has been noted before on isolated enzyme systems (4) and may be explained perhaps by the relatively slow rate of reaction of the iodoacetate with the enzymatic sulfhydryl groups.

The increase in oxygen uptake resulting from the addition of pyruvate to the heart slices was completely prevented by 0.001 *M* iodoacetate.

Fluoride

The endogenous respiration of rat heart slices was depressed by fluoride but the relation of this inhibition to the concentration of fluoride is complex, as may be seen from Table II. As the concentration of fluoride was increased, there was increasing inhibition until a maximal effect was reached at a concentration of approximately 0.02 *M*. However, if the concentration were further increased the inhibition became less, reaching a minimum at about 0.05 *M* fluoride. Further increase in concentration

TABLE II

Fluoride Inhibition of Respiration

Fluoride	No. of expts.	Q_{O_2}		Per cent inhibition	
		0–10′	0–60′	0–10′	0–60′
M					
0	2	−2.87	−2.48		
0.001	2	−2.59	−2.06	10	17
0	2	−2.87	−2.48		
0.005	4	−2.01	−1.69	30	32
0	2	−3.20	−2.69		
0.010	2	−2.08	−1.59	35	41
0	3	−2.96	−2.36		
0.020	3	−1.73	−1.22	42	48
0	3	−2.99	−2.32		
0.050	4	−2.30	−1.92	23	17
0	2	−3.11	−2.20		
0.100	2	−1.86	−1.66	40	25

again increased the inhibition of respiration. This phenomenon was so unexpected that we ran 3 more experiments comparing the actions of 0.02 *M* and 0.05 *M* fluoride; in all cases 0.02 *M* fluoride produced a greater inhibition than 0.05 *M* fluoride, although the difference was not as great as in the table, amounting to about 30% more inhibition with the 0.02 *M* (these experiments could not be included in the table because no controls without fluoride were run). The difference in effect between these two concentrations could be better observed later in the experiment than at the start. The results in all cases showed a difference in this direction, and therefore we believe that this phenomenon is real. More data must be obtained on this effect and on the action of fluoride on various enzyme systems before a reasonable explanation can be advanced.

Phlorizin

Phlorizin inhibited the endogenous oxygen uptake of rat heart slices as shown in Table III. This depression of respiration became progressively greater during the experiment, particularly with the 0.01 *M* phlorizin. The inhibitions with 0.01 *M* phlorizin were 61% at 10 min., 73% at 30 min., 83% at 50 min., and 91% at 70 min., thereafter the inhibition remaining fairly constant. On the other hand, in 0.002 *M* phlorizin the inhibition only dropped from 25% at 10 min. to 37% at 70 min., and at the end of 4 hr. was only 42%. Maximum inhibition is thus reached with phlorizin in about 70 min. A possible explanation for this development of inhibition may be the slow rate of penetration of phlorizin into the heart cells due to its relatively large molecular size.

TABLE III

Initial[a] *Inhibition of Respiration by Various Inhibitors*

Inhibitor conc.	Per cent inhibition of oxygen uptake					
	Malonate	Iodoacetate	Fluoride	Phlorizin	Pyrophosphate	Cyanide
M						
0.000001						14
0.00001						51
0.00005		23				
0.0001						68
0.0002		42				
0.0005		54				
0.001		70	10			74
0.002	11	76		25		
0.005	21	81	30		0	
0.01	32	95	35	61	21	
0.02	42	97	42		36	
0.05	62		23			
0.1	70		40			

[a] Based on oxygen consumption during period 0–10 min.

Pyrophosphate

Pyrophosphate was found to be a relatively weak inhibitor of the endogenous respiration of rat heart slices and to exhibit at high concentrations, like iodoacetate and phlorizin, a progressive inhibition with time. It may be compared with other inhibitors in Table III. Most of these experiments were run in calcium-free medium to avoid any interference of the pyrophosphate with calcium concentration; however, when calcium was present, almost identical results were obtained. In not one of the 21 experiments was there seen to be an increase in the respiration during the first hour, *i.e.*, a hump in the respiration curve, as was characteristic of malonate action. It would seem that, on this tissue, there is a difference in action between these two inhibitors, in this respect at least.

Cyanide

The action of cyanide on the endogenous respiration of rat heart slices is shown in Table III, from which it may be seen that, with respect to the concentration, it was the most potent inhibitor used in this study. The relation between cyanide concentration and inhibition is represented in Fig. 4. It is difficult to estimate accurately the fraction of the respiration that is passing through the cytochrome system, since, above 10^{-4} *M*, cyanide may be inhibiting systems other than cytochrome oxidase, but from the curves it may be judged that it is not far from 80% of the total respiration. The degree of inhibition increased during the experiments and, since here there is no question of the penetration of cyanide nor of its rapidity of action with the cytochrome oxidase, we may assume, therefore, that the depression of aerobic metabolism is damaging the tissue, perhaps in the same manner as anoxia (1).

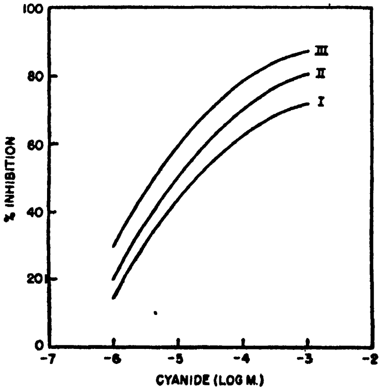

FIG. 4. Inhibition of respiration of rat heart slices by different concentrations of cyanide. Curve I: inhibition of initial (0–10 min.) respiration. Curve II: inhibition of respiration during the 1st hr. (0–60 min.). Curve III: inhibition of respiration during the 1st 3 hr. (0–180 min.).

DISCUSSION

There have been relatively few systematic investigations of the actions of metabolic inhibitors on the heart or its metabolism.

Malonate has been reported to inhibit the oxygen uptake of heart minces and slices (5, 6, 7), and our results also show inhibition of rat heart slice respiration by malonate at similar concentrations. Our results indicate an important role of succinic dehydrogenase in cardiac metabolism if it may be assumed that malonate has a specific action on this enzyme.

The production by malonate of a peak in the respiration curve of rat heart slices and the better maintenance of respiration in the presence of malonate may indicate an important phenomenon of cardiac metabolism. Two possible explanations of this phenomenon are immediately evident. There may be malonate-sensitive and malonate-resistant fractions of the endogenous respiration; the former might be of more importance in the heart slices at the start of the experiment but, due to changes taking place in the slices, it becomes of less significance and is partially replaced by the latter type of respiration. Thus, malonate would inhibit more potently at the beginning of the experiment than later. The alternative explanation would assume that the malonate itself is inducing this change in metabolic pattern in the heart slices. The fact that the inhibition is less marked when the malonate is added later in the experiment indicates that there is some intrinsic change in the metabolism that occurs independently of the inhibitor. On the other hand, the fact that certain concentrations of malonate will increase the respiration above the controls after the initial rise argues for an effect of the malonate itself in stimulating this type of respiration.

In rat heart slices fluoride at a concentration of 0.02 *M* was reported to act like iodoacetate at lower concentrations (8), but we have found that the maximum inhibition with fluoride is not over 50% in any concentration up to 0.1 *M* and that the action of fluoride in the higher concentration range involves factors that are absent in the case of iodoacetate.

SUMMARY

1. The actions of 6 enzyme inhibitors have been investigated on the respiration of rat heart slices.

2. It has been shown that malonate inhibits the initial endogenous oxygen consumption, but that with several concentrations of malonate the respiration subsequently increases to a peak which is greater than the control respiration at this time.

3. Fluoride inhibits the endogenous respiration, but the per cent inhibition does not increase regularly with increasing fluoride concentrations.

4. Iodoacetate, cyanide, phlorizin, and pyrophosphate produce increasing inhibition of the endogenous respiration with increasing concentration, and this inhibition progressively increases with time.

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Destruction of Influenza A Virus Infectivity by Urea [1,2]

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INTRODUCTION

Previous studies on the kinetics of the destruction by heating of infectivity for chicken embryos of PR8 influenza A virus have been reported (1). The present communication deals particularly with the kinetics of the destruction of infectivity in the presence of substantial concentrations of urea.

MATERIALS AND METHODS

Influenza A virus of the PR8 strain was used in these investigations. The virus was obtained from Dr. W. M. Stanley who had obtained it from Dr. Thomas Francis, Jr. Its history was described by Stanley (2). The preparation used in the studies herein reported was the pooled allantoic fluid from infected chicken embryos representing the eighth embryo passage in Pittsburgh. The urea used was of reagent grade.

The general procedure followed in this investigation was to add to 1/10 *M* phosphate buffer at pH 7 one part/100 of allantoic fluid containing virus, and sufficient urea to bring the urea concentration to some value between 0.5 and 3 molal. The solution containing urea and virus was then held in a water bath at some particular temperature. Samples were withdrawn periodically and 50% chicken embryo infectivity endpoints were determined. Red blood cell agglutinating ability was used as a criterion of infection. Details of the infectivity measurements and of the calculation of the endpoints were presented previously (1).

It was found in the earlier study that the infectivity was destroyed according to the law of a first order process; that is, the log of relative infectivity decreased linearly with time of heating. Similar results were obtained in the current investigation when the virus was heated in the presence of urea. The specific reaction rate in reciprocal minutes is 2.3

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[2] Contribution number 3p49 of the Department of Physics, University of Pittsburgh.

times the slope obtained when $\log_{10}$ of infectivity is plotted against time of heating in minutes.

Each rate constant was determined from numerous measurements of infectivity as a function of time of treatment. The slope of the straight line relating log of infectivity to time of heating was calculated by the method of least squares. The results of many experiments were used, for the endpoint of untreated virus, that is, for the end point at zero time. Specific reaction rates, k, were obtained by multiplying the slopes by 2.3. The standard error of k for each experiment was evaluated by multiplying the standard error of the slope by 2.3. The standard error of the slope, SE, was determined by means of the equation,

$$SE = \frac{\sigma_y}{\sigma_x} \frac{\sqrt{1 - r^2_{xy}}}{\sqrt{N - 2}}.$$

σ_y and σ_x represent the standard deviations of time and of logarithmic endpoint; r_{xy} is the correlation coefficient and N represents the total number of observations.

EXPERIMENTAL RESULTS

Experiments were carried out in which solutions in 1/10 M phosphate buffer at pH 7 containing infectious allantoic fluid at a concentration of 1 part/100 and urea at concentrations of 0.5, 1, 2 or 3 molal were heated at various temperatures between 36° and 50°C. The specific reaction rates and their standard errors are presented in Table I.

TABLE I

The Destruction of Influenza Virus Infectivity in the Presence of Urea

Urea conc.	Temperature	k	Standard error of k
molal	°C.	*min.*$^{-1}$	
0.5	41	0.14	0.02
1.0	38	0.11	0.01
1.0	41	0.28	0.02
1.0	46	0.60	0.05
1.0	50	2.28	0.16
2.0	36	0.14	0.02
2.0	38	0.39	0.05
2.0	41	0.74	0.14
2.0	46	2.21	0.14
3.0	41	2.76	0.46

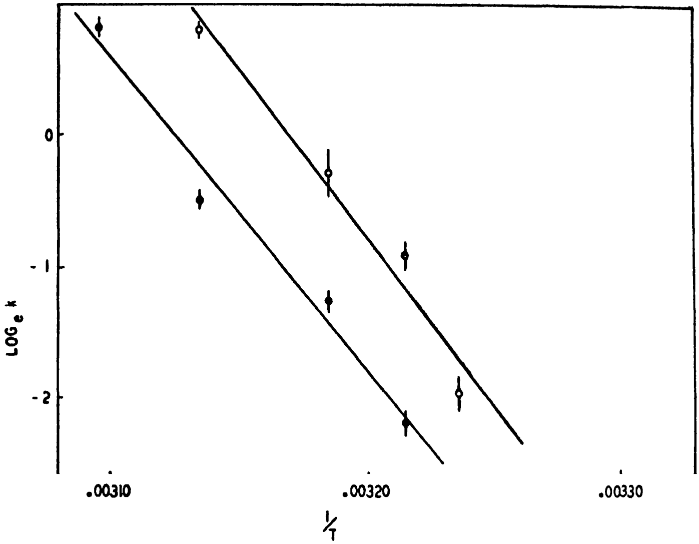

FIG. 1. Natural log of specific reaction rate for the destruction of PR8 influenza A virus infectivity plotted as a function of reciprocal of absolute temperature: ○—2 molal urea; ●—1 molal urea; vertical lines represent range of ± one standard error.

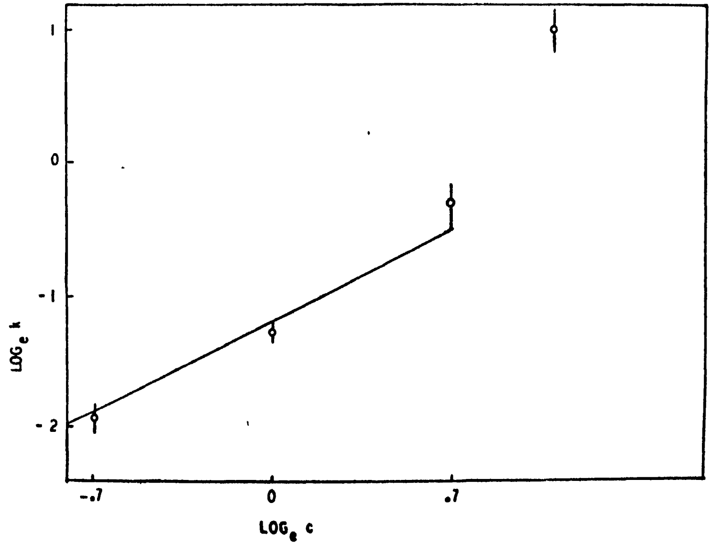

FIG. 2. Natural log of specific reaction rate for the destruction of PR8 influenza A virus infectivity plotted against natural log of concentration of urea expressed in terms of molality. Vertical lines represent range of ± one standard error.

Some of the data can be grouped to show the effect of temperature upon the rate of destruction of influenza virus activity in the presence of urea. In Fig. 1, $\log_e$ of specific reaction rate is plotted against reciprocal of absolute temperature for urea concentrations of 1 and 2 molal, respectively. The circles represent the appropriate data taken from Table I. The vertical lines represent the ranges corresponding to plus or minus one standard error for each datum.

The remainder of the data show the effect of urea concentration upon the specific reaction rate at a constant temperature of 41°C. In Fig. 2, $\log_e$ of k is plotted against $\log_e$ of molal concentration of urea. It can be seen that a straight line relationship affords a reasonable approximation of the data obtained for 0.5, 1 and 2 molal urea solutions. The rate obtained with a 3 molal urea solution, however, is much higher than would be indicated by this linear relationship.

DISCUSSION

It was shown previously (1), on the basis of twenty individual measurements covering the temperature range from 40° to 56°C., that, when $\log_e$ of specific reaction rate for destruction of influenza virus activity in 0.1 M phosphate buffer at pH 7 was plotted against reciprocal of absolute temperature, according to the Arrhenius equation, a straight line relationship was obtained within experimental error. Accordingly, the data obtained for the destruction of virus infectivity in the presence of 1 molal and 2 molal urea were also plotted according to the Arrhenius equation in Fig. 1. From the slopes of these two straight lines values for energy of activation of 48,000 and 52,000 cal./mole were obtained for the reactions in 1 and 2 molal urea, respectively.[3] On the basis of the Eyring concept of absolute reaction rates,[4] values for the entropy of activation were calculated from these graphs to be 90 and 100 cal./mole degree for the reaction in 1 and 2 molal urea, respectively. The energy and entropy of activation for the destruction of infectivity in the absence of urea were calculated in a previous study to be 34,000 cal./mole and 39 cal./mole degree (1).

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[3] By a method analogous to that used in computing the standard error of k, the standard error of the energy of activation for the inactivation of influenza virus in urea can be shown to be of the order of magnitude of 6000 cal./mole. Thus, it is obvious that the difference between the energy of activation in 1 molal and in 2 molal urea is not significant. Of more importance, however, is the question whether the energy of activation in the presence of either 1 or 2 molal urea differs significantly from the 34,000 cal./mole obtained for the inactivation in the absence of urea. It can be shown by standard procedures of statistical inference that the probability that each of the values obtained in the presence of urea does not differ significantly from 34,000 is of the order of 0.1. Thus, the probability that both of these values could have been obtained, if the energy of activation in the presence of urea is not really higher than that in the absence of urea, is of the order of .01. On the basis of these considerations, it seems reasonably safe to conclude that the energy of activation for the inactivation of influenza virus in the presence of urea really is higher than that for the inactivation in the absence of urea.

The effect of urea on the destruction of the red blood cell agglutinating ability of PR8 influenza A virus had been studied previously (3). In the absence of urea, the energy of activation was approximately 110,000 cal./mole (4), but the energy of activation decreased as the urea concentration increased until a value of approximately 40,000 cal./mole was obtained in a 4 molal urea solution. The entropy of activation was also decreased by the action of urea. Eyring and Stearn (5) calculated the energy and entropy of activation for the denaturation of egg albumin in the absence of urea and in the presence of urea. In this case, too, very marked decreases in both were observed when the solvent was changed from water to urea solution. In fact, negative values for both energy and entropy of activation were obtained for the reaction in the presence of urea. The energy of activation for the disintegration of tobacco mosaic virus protein at high temperatures was found (6) to be about 150,000 cal./mole. Boyd and Eberl (7) recently calculated the entropy of activation for this reaction from the data of Lauffer and Price (6) to be around 350 cal./mole/degree.

The disintegration of tobacco mosaic virus in the presence of urea

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[4] The Eyring concept of absolute reaction rates, in slightly simplified form, relates the specific reaction velocity constant, k, to the heat change, $\Delta H^\ddagger$, and the entropy change, $\Delta S^\ddagger$, when a particle goes from a normal state to an activated or reactive state, according to the equation:

$$k = \frac{RT}{Ah} e^{\Delta S^\ddagger/R}\, e^{-\Delta H^\ddagger/RT},$$

R is the gas constant, T is the absolute temperature, A is Avagadro's constant, and h is Planck's constant. The energy of activation, E, in the familiar Arrhenius equation is

$$R\,\frac{d \ln k}{d\,(1/T)},$$

and is thus equal to $\Delta H^\ddagger + RT$.

For reactions at ordinary temperatures, $\Delta H^\ddagger$ and E differ by about 600 cal./mole. E, for most protein denaturation and related reactions, is of the order of 100 times this value. Since the error inherent in the measurement of E in most protein denaturation reactions is of the order of several per cent, the value of E was used as an estimate of $\Delta H^\ddagger$ in the present discussion. In practice, this is the equivalent of treating $\Delta H^\ddagger$ and E as synonyms. All value for E and $\Delta S^\ddagger$ were rounded to two significant digits.

is a complex reaction, for it proceeds with minimum velocity at room temperature and at higher velocities at temperatures both below and above room temperature (8, 9). Other plant viruses (10) and some proteins behave similarly. This has been explained (9) by postulating that at least 2 parallel reactions are involved, one of which speeds up as temperature is increased and one of which speeds up as temperature is decreased. On the basis of the assumption that there are only 2 such parallel reactions, the data of Lauffer (9) indicate that one of the reactions in the presence of 6 molar urea has an energy of activation of about 27,000 and an entropy of activation of 20, while the other has corresponding values of about −19,000 and −160. Thus, in the case of the disintegration of tobacco mosaic virus protein, urea decreases both the energy of activation and the entropy of activation.

It is apparent from the foregoing considerations that, in denaturation reactions studied previously, the effect of urea was to decrease both energy of activation and entropy of activation. Since urea acts to increase both in the case of the destruction of PR8 influenza A virus infectivity, this reaction constitutes an exception to the previously observed pattern.

Fig. 2 shows the relationship between log of specific reaction rate constant and log of urea concentration. A linear relationship could be interpreted to mean that the reaction rate is directly proportional to the urea concentration raised to some exponent, the value of which is equal to the slope of the straight line. The data obtained with the 3 more dilute urea solutions fall on a straight line within the measured experimental error. The slope of this straight line is approximately 1. This can be interpreted to mean that one molecule of urea combines with one molecule of the virus as one of the steps in the activation process. The rate constant obtained in 3 molal urea does not fit this straight line relationship; however, it is not surprising that this value deviates from the trend for the more dilute solutions, for many of the thermodynamic properties of concentrated solutions differ remarkably from the trends which apply to dilute solutions.

SUMMARY

The kinetics of the destruction of PR8 influenza A virus infectivity in the presence of concentrated solutions of urea was investigated. The destruction of infectivity follows the course of a first order re-

action. The energy and entropy of activation for the process which takes place within the temperature range, 36 to 50°C., were determined and found to be 48,000 cal./mole and 90 cal./mole/deg, respectively, when the urea concentration was 1 molal, and 52,000 cal./mole and 100 cal./mole/deg., respectively, when the urea concentration was 2 molal. When the log of the reaction rate at 41°C. was plotted against the log of urea concentration, a straight line with a slope of unity was obtained for urea concentrations between 0.5 and 2.0 molal. The rate obtained with 3.0 molal urea solution did not fit this relationship.

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Letters to the Editors

Utilization of Choline and Betaine Methyl in the Guinea Pig

In vitro studies have shown that in all animals tested betaine and dimethylthetin increase methionine formation from homocysteine in liver homogenates (1). In those animals which have choline oxidase, choline is also effective. The guinea pig does not have choline oxidase and does not form methionine from choline and homocysteine under these conditions (2). This is in agreement with the finding by Handler that guinea pigs do not show signs of choline depletion when fed glycocyamine or nicotinamide (3).

The *in vivo* availability of choline and betaine for methylation in the guinea pig was checked by injecting these compounds labeled in the methyl groups with C^{14} as described by du Vigneaud and coworkers (4). Animals on a stock diet were injected intraperitoneally with a total of 1 millimole of the test substance containing 1.2×10^6 counts over a 3-day period. The respiratory CO_2 was collected and analyzed as $BaCO_3$. The liver, kidney, and the washed intestinal tract were frozen, ground, and exhaustively extracted with boiling alcohol and with

TABLE I

Utilization of Labeled Methyl by the Guinea Pig

Compound isolated	Compound injected		
	Choline	Betaine	Ratio of betaine to choline utilization
Respiratory CO_2 (infinite thickness 3.14 cm.^{2})	22.5 cts./min.	102.5 cts./min.	4.6
Liver, kidney, and intestinal tract proteins (infinite thickness 3.14 cm.^{2})	52.6 cts./min.	238 cts./min.	4.6
$(CH_3)_4I$ from carcass proteins	4.5 cts./min./mg.	21.5 cts./min./mg.	4.8

trichloroacetic acid. The rest of the carcass was treated in a similar manner and analyzed for S-methyl groups as described by Simmonds *et al.* (5). The activities of the three fractions are shown in Table I.

It is clear that choline methyl is labile in the guinea pig, but it is quantitatively much less active than betaine methyl.

In vitro experiments on guinea pigs (2) do not support the view that choline methylates homocysteine directly or after oxidation to betaine. Unpublished experiments suggest an alternate mechanism whereby choline reacts with homocystine, as implied in the finding that homocystine plus choline is more effective than homocysteine plus choline in the formation of creatine from glycocyamine (6).

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A Method for the Determination of the Amino Acid Sequence in Peptides

A simple procedure has been worked out for the stepwise degradation of peptides. The underlying principle has earlier been employed by Abdherhalden and Brockmann (1), who observed that the phenylureido derivate of a peptide was split by concentrated methanolic HCl at elevated temperature into the phenylhydantoin of the amino acid residue carrying the free α-amino group and a peptide with one amino acid residue less than the original peptide. The main shortcoming of their method seems to originate from the fact that during the rather vigorous treatment required for the desired splitting, the other peptide bonds are also split to a small but definite extent.

In our procedure this difficulty has been overcome by carrying out the cleavage in an anhydrous medium and also by employing the phenylthioureido derivatives, which form hydantoins more easily. The procedure has been worked out as a micromethod, the requirements of substance being approximately 10 mg. of amino acid residue per peptide bond.

The peptide is reacted with phenylisothiocyanate in pyridine-water (1:1 by vol.) at 40°C. and pH 8.6. The pyridine is then extracted with benzene, and the phenylthioureido peptide precipitated in acetic acid as its silver salt and thoroughly dried. The dry thioureido derivative is treated with an anhydrous, saturated solution of HCl in nitromethane, which causes the hydantoin to be split off. In all cases investigated this reaction is extremely rapid, even at room temperature, and is unaccompanied by a splitting of other peptide linkages. The split products are separated and the thiohydantoin hydrolyzed to the corresponding amino acid, which is identified on paper strip chromatograms. The whole procedure is then repeated on the remaining peptide. The method has been successfully applied to the following synthetic peptides: alanylglycine, leucylglycine, leucyltyrosine, leucylglycylglycine and alanylleucylglycine.

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Borrelidin, a New Antibiotic with Anti-borrelia Activity and Penicillin Enhancement Properties

A new antibiotic has been isolated in crystalline form from the culture medium of a new Streptomyces species named *Streptomyces rochei.* The antibiotic shows an unusual degree of specificity *in vitro.* It is highly active against *Sarcina lutea* and certain micrococci, but against most other common test bacteria it is relatively inactive. A unit was defined as 0.06 γ of the crystalline compound.

The new antibiotic is rather toxic and without effect in experimental bacterial and viral infections of mice. However, it shows a high and specific *in vivo* activity against different strains of *Borrelia*, the relapsing fever spirochete, suggesting "Borrelidin" as its name. These chemotherapeutic studies were carried out by Buck, Farr and Schnitzer and are reported in detail elsewhere (1).

The specific anti-spirochetal activity of borrelidin led to the assumption that it might be related to one of the enhancement factors claimed to be present in impure penicillin; in particular, to the factor responsible for the observed higher activity of impure penicillin over crystalline penicillin G against the syphilis spirochete (2). This assumption was verified experimentally by the preparation of concentrates from inactivated impure penicillin, which exhibit the specific *in vitro* activity of borrelidin against *Sarcina lutea* and its high specific *in vivo* activity against *Borrelia*. Concentrates with similar *in vitro* and *in vivo* activities were also obtained from corn steep and "Clarase" enzyme preparations. The latter was investigated because of the claim by Hobby *et al.* (3) that it contains a penicillin-enhancing substance. Various "Clarase" samples differed widely in their borrelidin-like activity, ranging from 10 to 2000 *Sarcina lutea* plate units/g. Corn steep samples contained between 3 and 60 units/g., and inactivated crude penicillin preparations 0.2 to 35 units/mg.

These observations led to the study of crystalline borrelidin as an enhancement factor for penicillin. As reported by Grunberg *et al.* (4), the enhancement effects observed are similar to those described for the factors present in impure penicillin (2,3,5). However, certain differences were also noted. Furthermore, none of the penicillin by-products thus far isolated in pure form has been found to be identical with borrelidin.

Borrelidin is produced when *Streptomyces rochei* is grown on a variety of media, in stationary or aerated submerged culture. Particularly suitable are media containing soybean meal, sodium acetate and phosphate, which yield potencies up to 500 units/ml. It can be isolated from culture solutions by extraction with butyl acetate at pH 7. The butyl acetate concentrate is purified by treatment with bentonite clay which adsorbs impurities. The residue, obtained after evaporation of the filtrate, is treated in benzene solution with bentonite clay, which in this solvent adsorbs borrelidin. The borrelidin is then eluted with methanol. The eluate is dissolved in ether, and, after removal of inactive material by extraction with phosphate buffer of pH 7, the antibiotic is extracted with dilute NaOH. The alkali solution is acidified, the borrelidin extracted with benzene, and the benzene solution concentrated. On standing, crystals of borrelidin are deposited. Recrystallization from benzene gives pure borrelidin, m.p. 145–146°C., $[\alpha]_D^{27} = -28°$ in ethanol.

Borrelidin is an acid. A crystalline methyl ester (m.p. 153–154°C.) and a crystalline *p*-nitrobenzyl ester (m.p. 161°C.) have been obtained. The methyl ester gives a crystalline diacetyl-(m.p. 190°C.) and a crystalline di-*p*-nitrobenzoyl-(m.p. 157°C.) derivative. The microanalytical results thus far obtained indicate an empirical formula $C_{28}H_{43}NO_6$ for borrelidin. The ultraviolet absorption spectrum in isopropanol shows a maximum at 256 mμ ($E_{1\,cm.}^{1\%} = 550$), indicating a site of conjugated unsaturation in its molecule.

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Sulfocholine in Methionine Synthesis[1]

Sulfocholine (β-hydroxyethyldimethylsulfoniumchloride) has been prepared according to the method of Renshaw *et al.* (1) and tested as a possible precursor of dimethylthetin, a compound which rapidly methylates homocysteine in liver homogenates (2).

As shown in Table I, sulfocholine rapidly forms methionine from homocysteine under aerobic conditions but not under anaerobic conditions. Sulfocholine also increases the oxygen consumption of enzyme preparations which are capable of oxidizing choline to betaine. These experiments suggest that the methyl groups of sulfocholine, like choline (4), may not be labile until the alcohol group has been oxidized. If, like choline, the alcohol group of sulfocholine is oxidized to a car-

[1] Aided by a grant from the U. S. Public Health Service.

boxyl group, the product of the oxidation is dimethylthetin, which is the most effective methylator of homocysteine yet found (2). Methylmercaptoacetic acid, which is demethylated dimethylthetin, has been detected by paper chromatography at the end of the reaction.

Recently, Maw and du Vigneaud (5) concluded from growth experiments with rats on methionine-free diets that the methyl groups of sulfocholine were not labile but did prevent haemorrhagic kidneys. Because these experiments were not in agreement with our *in vitro* studies, sulfocholine was synthesized by the new method described by these workers. This product was biologically equivalent to that prepared by the method of Renshaw in promoting methionine formation *in vitro*.

TABLE I

Methylation of Homocysteine in Rat Liver Homogenates

Substrate	100% N_2		4% O_2 + 96% N_2	
	Total methionine mg.-%	Net increase	Total methionine mg.-%	Net increase
DL-Homocysteine	3.5		1.7	
DL-Homocysteine + choline	3.5	−0.2	2.6	+0.9
DL-Homocysteine + betaine	5.5	+2.0	3.6	+1.9
DL-Homocysteine + sulfocholine	3.7	+0.2	3.5	+1.8

All compounds 25 mg.-% in a total volume of 4 ml. containing 1 ml. of rat liver homogenized in 2 parts of 0.02 *M* phosphate buffer. Methionine determined by the McCarthy and Sullivan reaction (3). Average of two determinations; probable error 0.15 mg.-%.

The apparent disagreement between the *in vivo* and the *in vitro* experiments may be reconciled if one assumes that sulfocholine is too toxic to be the sole source of methyl in the diet but is capable of becoming a methyl donor at low concentrations. That this explanation is correct is suggested by the results reported here and the slightly positive weight gains shown in the growth experiments at low levels of sulfocholine before the toxic symptoms became evident.

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Coenzyme A and Citrate Formation in Homogenates of Heart Ventricle from Normal and Pantothenic Acid-Deficient Ducklings [1]

The demonstration by Lipmann and his coworkers that coenzyme A contains pantothenic acid in a conjugated form (1) and accounts for all of this vitamin in native tissues (2) has led to an extensive search for its functions in metabolism. Thus far it has been shown that this coenzyme plays a role in the acetylation of aromatic amines (3), in acetylcholine synthesis (4), in acetyl phosphate synthesis (5), and in acetoacetate synthesis (6).

The derangement of pyruvate metabolism in pantothenic acid-deficient bacteria (7) and animal tissues (8) has been shown to correlate with depressed bacterial (9) and tissue coenzyme A levels (10). By means of two radioisomers of pyruvate, labeled in the carboxyl and carbonyl groups respectively, it has recently been shown (11) that, while the rate of decarboxylation of pyruvate is decreased in homogenates of heart ventricle from pantothenic acid-deficient ducklings, the rate of oxidation of the α-carbon atom is depressed significantly more. The rate of oxidation of acetate is also decreased in homogenates of pantothenic acid-deficient heart muscle (11) and in pantothenic acid-deficient yeast (12). These data suggest that the role of coenzyme A in pyruvate and acetate oxidation is concerned with an early step in acetate catabolism, possibly the condensation of the active C_2-moiety with oxalacetate.

In this communication we should like to report the results of a study of citrate synthesis in barium-inhibited homogenates of heart ventricle from pantothenic acid-deficient and normal pair-fed ducklings. Experimental and pairfed control birds were raised on diets

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previously described (10), and homogenates of heart ventricle prepared by homogenizing the chilled tissue with 9 volumes of cold isotonic KCl. Homogenate in the amount of 0.5 ml. was added to prepared Warburg vessels containing 2.5 ml. of cold medium, with substrate and coenzyme additions as indicated in the accompanying table. At the end of 30 min. of incubation at 37°C. the tissue reactions were stopped with trichloroacetic acid and citrate determined spectrophotometrically on the protein-free filtrate. The results of a typical experiment, expressed in terms of $+ Q_{\text{Citrate}}$ (μl. of citrate formed/mg. dry weight of homogenate/hr.), are shown.

TABLE I

Homogenate	$+Q_{\text{Citrate}}$					
	(1)	(2)	(3)	(4)	(5)	(6)
	No added substrate	Pyruvate 5 m*M*/l.	Fumarate 5 m*M*/l	Pyruvate +fumarate ea. 5 m*M*/l.	Pyruvate +fumarate +coenzyme Fr.[a]	Pyruvate +fumarate +Co. A[b]
Pantothenic acid-deficient	0.0	0.2	0.2	0.7	1.8	1.3
Pair-fed control	0.0	0.3	0.2	1.4	2.5	2.0

Warburg flasks contained a total of 3.0 ml. of medium with KOH in the center wells. Gas phase: air. Temperature: 37°C. Medium: KCl 0.154 *M*, $BaCl_2$ 0.01 *M*. Pyruvate and fumarate were added at 5 m*M*/l whether singly or together. Coenzyme A, when present, was added in amounts of 20 acetylation units per flask. The coenzyme fraction indicated in Col. 5 was a crude preparation of coenzyme A containing 12 units/mg. The "Co A" referred to in Col. 6 was a purified fraction containing 110 units/mg. Thus, the total solids of coenzyme supplement in the first case was 1600 γ per flasks; in the second 200 γ per flask. Period of incubation: 30 min. Reactions stopped with 0.2 ml. of 100% trichloroacetic acid and the citrate determined on a Beckman spectrophotometer by the pentabromoacetone procedure.

[a] Crude coenzyme fraction prepared by precipitating a concentrated trichloroacetic acid filtrate of boiled rabbit liver with 10 volumes of acetone.

[b] We are greatly indebted to Dr. Fritz Lipmann for a sample of one of his purified preparations of coenzyme A containing 100 units/mg.

These data lend support to the supposition that coenzyme A is involved in the initial condensation of the tricarboxylic acid cycle. The apparent superiority of the crude preparation of coenzyme A over the more completely purified one of the same acetylation unitage in stimulating citrate synthesis is probably due to its content of other coenzyme (cocarboxylase, DPN, *etc.*) which would hasten the conversion of the substrates used in these experiments to the immediate reactants of the condensation reaction, *i.e.*, fumarate to oxalacetate and pyruvate to active acetate. Since pyruvate is the source of the C_2-moiety in these experiments, a possible ancillary effect of coenzyme A in facilitating the transformation of pyruvate to active acetate is not ruled out.

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The Problem of Heterocarboxylic Metabolites

The problem of the necessity of CO_2 for the growth of bacteria has been examined by us in 1946–1947. The hypothesis was that CO_2 is a necessary building block in the synthesis of one or several essential metabolites for which we suggested the term "heterocarboxylic essential metabolites."

The growth rate of *Escherichia coli* was determined under controlled CO_2 pressure. It was found that glutamic acid does not change the

[3] This work was done during the tenure of a Research Fellowship of the American Heart Association.

growth rate under optimal pressure of CO_2. If the CO_2 tension is lowered the growth rate decreases; glutamic acid and other C_4 or C_5 dicarboxylic acids compensate this effect partially. But at very low CO_2 tensions C_4 and C_5 dicarboxylic acids are without action. We concluded that other heterocarboxylic metabolites must be essential for growth. The evidence suggests that such substance or substances are present in yeast extracts. They are not yet identified.

Ajl and Werkman (1948) have taken up this problem again and announced that glutamic acid, as well as other C_4 and C_5 dicarboxylic acids would "substitute" for CO_2, and permit growth in its absence.

We wish to make the following points:

1. We have followed the growth curve and estimated growth rates, whereas Ajl and Werkman have determined the "turbidity after 18 hr."
2. In our experiments, 20 times more CO_2-free air was passed per unit time, as in Ajl and Werkman's experiments
3. These differences in methods may explain why Ajl and Werkman have failed to recognize the fact that CO_2 is not essential for the synthesis of C_4 and C_5 dicarboxylic acids only, but also for the formation of other heterocarboxylic metabolites.

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An Unidentified Factor in Pancreas Essential for the Sustained Growth of *Trichomonas vaginalis*

The growth-stimulating properties of pancreatic extracts have been reported previously for tissue cultures (1,2) and for mice (3). In attempts to replace BBL "trypticase" from the complete medium of Sprince and Kupferberg for the protozoan, *Trichomonas vaginalis* (4,5,6), it was observed that pancreatic extracts were very effective in stimulating growth of this organism. This effect was shown to be due to some factor (*s*), other than all the known growth factors including strepogenin (7) and vitamin B_{12}, and the ordinarily available amino acids. Certain antifatty-liver fractions (8) of pancreas were especially rich in the active material. Growth, however, could not be obtained with a mixture of methionine and choline (9).

EXPERIMENTAL

The organism used was a bacteria-free culture of *Trichomonas vaginalis*, Strain No. 2. The testing medium was essentially the medium of Sprince and Kupferberg (4) with the following modifications: phosphate buffer (salt solution A of *L. casei* media) was added to give a concentration of 50 mg. of phosphate/100 cc. of medium (10); Merck's crystalline vitamin B_{12}, to give a concentration of 0.02 γ/100 cc. of medium (11). Five-tenths cc. of sterile undiluted intact serum adjusted to pH 6.0 were used per tube in place of sterile diluted serum. Details for growth and preparation of inocula have been reported in previous papers (4,5,6). In the present work, 0.05 cc. of washed inoculum was used for the initial 72 hr. culture; 0.07 cc. was used for the two subsequent 48 hr. serial transfers.

TABLE I

Replacement of BBL "Trypticase" by Pancreas Fractions
(Each value is the average of duplicate determinations.)

Material added	Growth of *Trichomonas vaginalis* expressed in number of cells/mm.^{3} at end of 2 serial transfers from the initial 72-hr. culture
Basal medium (contg. 5% serum) (control)	0
Basal medium + 2.0% BBL "trypticase" (control)	2535
Basal medium + 0.01% BBL "trypticase"	0
Basal medium + 0.05% BBL "trypticase"	115
Basal medium + 0.01% Lipocaic (Lilly)	670
Basal medium + 0.05% Lipocaic (Lilly)	1590
Basal medium + 0.01% Purified Liver Extract (Lilly)[a]	200
Basal medium + 0.01% triple strength pancreatin (Wilson)	130
Basal medium + 0.01% Pancreas "Al" fraction (Sharp-Dohme)	360
Basal medium + 0.05% Pancreas "Al" fraction (Sharp-Dohme)	1550
Basal medium + 0.01% cryst. chymotrypsin (Worthington)	0
Basal medium + 0.01% cryst. trypsin (Worthington)	0
Basal medium + 0.01% cryst. insulin (Lilly)	0
Basal medium + 2.0% Parenamine (Stearns)	0
Basal medium + 2.0% Parenamine + 0.1% insulin	0
Basal medium + 0.05% cryst. methionine	0
Basal medium + 0.05% cryst. choline	0

[a] Solution liver extract purified, Lilly, 10 injectable U.S.P. Units of antipernicious-anemia principle/cc. 1 cc. = 127.2 mg. dry wt. approx.

In testing for the activity of various materials, "trypticase" was omitted from the basal medium and various substrates under test were added. The tubes were autoclaved, and the sterile serum was then added in the usual way. Table I presents data to indicate the activity of these materials in replacing "trypticase."

The active material was found to be water-soluble, dialyzable, and stable to autoclaving at 15 lbs. for 15 min. It could not be replaced by Merck's crystalline vitamin B_{12} in concentrations as high as 10 γ/100 cc. of medium. Lilly's Lipocaic, which was the most active fraction thus far tested, could be demonstrated to effect detectable growth at 25 γ/cc. of final medium. This could be shown to be about 20 times as active as BBL "trypticase," which gave detectable growth at 500 γ/cc. Further details concerning the active factor (*s*) will be published in a forthcoming paper.

ACKNOWLEDGMENTS

We wish to thank Dr. Edward D. Campbell of the Lilly Research Laboratories for generous amounts of Lipocaic and Cristalline insulin. To Dr. David K. Bosshardt of Sharp and Dohme, we are indebted for a large gift of Pancreas Fraction "Al." We are especially grateful to Mr. David Hendlin of Merck Laboratories for generaous amounts of vitamin B_{12}.

CONCLUSIONS

Pancreas contains an unidentified factor (*s*) essential for the growth of the protozoan, *Trichomonas vaginalis*. The Lipocaic fraction (Lilly) was the most active fraction thus far tested. Detectable growth could be demonstrated at a concentration of 25 γ/cc. of medium. None of the known growth factors, including strepogenin (from insulin), Merck's crystalline vitamin B_{12}, amino acids of fortified casein hydrolyzate (Parenamine), nor the lipotropic substances methionine and choline could replace the active material from pancreas.

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Mechanism of the Growth-Promoting Effect of Ascorbic Acid on *Lactobacillus leichmannii* and the Reduction of Oxidation Products of Vitamin B_{12} [1]

Several microorganisms that utilize either vitamin B_{12} or thymidine for growth have been shown also to repsond to ascorbic acid (1,2,3). In view of the improbability of vitamin C replacing vitamin B_{12}, or the active desoxyribosides (3,4), its effect on the growth of *L. leichmannii* (ATCC 4797) has been studied. Each group that noted the effect of ascorbic acid used a medium that contained a charcoal-treated, tryptic digest of casein. Using an amino acid medium,[2] we find that ascorbic acid is without activity as a growth factor; however, the addition of trypsinized casein enables ascorbic acid (0.01–0.1%), particularly when autoclaved with the medium, markedly to augment the inherent microbiological activity of the hydrolyzate. This effect of ascorbic acid is shared by glucoascorbic acid, thioglycolic acid, glutathione and

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[1] This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[2] The medium was that of Skeggs *et al.* (2), except that nitrogen was supplied by the following amino acids (each at 2 mg./10 ml.): DL-alanine, DL-aspartic acid, L-arginine HCl, L-cystine, L-glutamic acid HCl, glycine, L-histidine HCl, L-hydroxyproline, DL-isoleucine, DL-lysine HCl, DL-methionine, DL-norleucine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tryptophan, L-tyrosine, DL-valine; in the experiments with crude trypsin without casein the level of each was 3.4 mg./10 ml. For inoculation, one drop of a twice-washed saline suspension (Klett reading of about 10) of organisms was used; this was prepared from a 7-hr. culture, made from a 24-hr. culture, each in basal medium containing 0.05 ml. of 10-unit liver extract (Lederle) per 10 ml. Initial transfer was made from 1% tryptose-milk.

sodium formaldehydesulfoxylate; the activity of the tryptic digest also is increased by autoclaving it separately (pH 7, 120°C., 15 min.) with such reducing agents.

A digest of "vitamin-free" casein ("Labco") (5–250 mg./10 ml. of medium), made with twice-recrystallized trypsin (by itself essentially

Growth-Promoting Effect of Tryptically Digested Casein and of Crude Trypsin

Addition/10 ml. medium[2]			Klett readings[a] 16 hr. 37°C.
Casein digest[b]	Vitamin B_{12}	Ascorbic acid	
mg.	*mγ*	*mg.*	
			18
		10	17
	0.1		74
	0.1	10	79
	0.5		173
	0.5	10	189
10			48
10		1	145
10		3	167
10		10	167
25			102
25		1	254
25		3	258
100			264
100		1	325
100		3	345
100	0.5		305
100	0.5	10	350

Addition/10 ml. medium[2]			Klett readings[a] 16 hr., 37°C.	
Casein digest[c]	Trypsin[4]		Without ascorbic	With ascorbic
	Unmodified *mg.*	Alkali-treated[d] *mg.*		
mg.				(*5 mg.*)
5			41	85
15			126	204
50			258	315
150			335	490
			19	24
	0.4		59	96
		0.4	43	49
		0.4(0.17)	47	63
	4.0		272	320
		4.0	147	176
		4.0(1.7)	187	254
	40.		555	560
		40.	455	510
		40. (17.)	455	480

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[a] Turbidity readings at 16 hr. approximate 60% of the values at 72 hr.

[b] "Vitamin-free" casein ('Labco') prepared with crude trypsin,[4] according to the method of Skeggs *et al.* (2); crude trypsin—2% of weight of casein.

[c] "Vitamin-free" casein ("Labco") digested 64 hr. with twice-recrystallized trypsin (Worthington—50% $MgSO_4$); final amount of actual trypsin—2% of weight of casein; this hydrolyzate was not treated with charcoal.

[d] Autoclaved (120°C.) at pH 10 for 1.5 hr.; the figures in parentheses indicate the initial amount of vitamin B_{12} (in mγ) added prior to autoclaving with alkali.

inactive), promotes vigorous bacterial growth in the absence of added vitamin B_{12}[3] and the activity is increased by ascorbic acid treatment. In the absence of casein, impure trypsin[4] of the type widely used in preparing casein hydrolyzates also is highly active (0.5–25 mg./10 ml. of medium). The activity is altered only slightly by heating (100°C.) at pH 10 for 0.5 hr., and autoclaving (120°C.) at pH 10 for 1.5 hr. reduces the activity by only about one-half; the remaining activity is increased by ascorbic acid treatment. Vitamin B_{12} added prior to such vigorous treatment is not completely inactivated and the residual activity is increased by ascorbic acid (see table). Autoclaving vitamin B_{12} in water at pH 10 for 0.5 hr. reduces its microbiological activity by over 99%, but addition of acid-hydrolyzed casein (200 mg. in 10 ml.) reduces the inactivation of 0.1 γ of the vitamin to about 65%; however, on autoclaving for 1.5–2 hr., the inactivation exceeds 98%. Under each of these conditions the residual microbiological activity is increased by treatment with ascorbic acid. Autoclaving of thymidine at pH 10 for 1.5 hr. causes no loss of microbiological activity and the potency is not augmented by ascorbic acid.

The material in "vitamin-free" casein and in crude trypsin that resists inactivation by prolonged autoclaving at pH 10 could consist in part of desoxyribosides, but preliminary data suggest that dexosyribonucleic acids or other products of its degradation may be involved. The substance which diminishes in activity on autoclaving at pH 10, the activity of which is restored partially by treating with ascorbic acid or other reducing agents, behaves like vitamin B_{12}. It is suggested that thermal inactivation of the vitamin at alkaline reactions yields oxidation products of which a portion can be converted by reduction to microbiologically more active forms. The apparent microbiological activity of ascorbic acid may be attributed to the occurrence of oxidation products of vitamin B_{12} in impure trypsin and in charcoal-treated, tryptically digested "vitamin-free" casein.[5]

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[3] Vitamin B_{12} was used in the form of concentrates (from several sources) or as pure crystalline material (Merck).

[4] Trypsin (1:250), Difco Laboratories, Detroit, Michigan.

[5] Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., have just reported [*Federation Proc.* **8**, 257 (1949)] that thioglycolic acid and ascorbic acid protect vitamin B_{12} against destruction during autoclaving of assay media containing the vitamin.

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Lysis of *Clostridium perfringens*

In the course of enzyme studies with washed "resting" cells of *Clostridium perfringens* it was observed that, under certain conditions, the cells underwent rapid lysis. Investigation of this phenomenon showed that the lysis was initiated by inorganic phosphate. Cell suspensions containing 15 mg. (dry weight) of cells/ml. were lysed to the extent of 80% in 60–80 min. at 45°C.

TABLE I

Lysis of Washed Cells of Clostridium perfringens in the Presence of Inorganic Phosphate and Other Substances

Suspensions contained 15 mg. cells (dry weight)/ml. Incubation at 45°C. for 60 min. Lysis was measured in a turbidimeter. The pH was 7.2 in all cases.

Suspending medium	Cells from 6-hr. culture	Cells from 18-hr. culture
	per cent lysis	*per cent lysis*
Water	0	0
Phosphate 0.005 *M*	53	67
Phosphate 0.015 *M*	78	84
Phosphate 0.030 *M*	47	43
Adenylic acid (muscle) 50 γ	72	77
Sodium pyrophosphate 0.015 *M*	10	6
Sodium pyrophosphate 0.030 *M*	8	0
Adenylic acid (yeast) 50 γ	0	0
Adenosine 50 γ	0	0
Adenine sulfate 50 γ	0	0
Guanylic acid 50 γ	0	0
Borate buffer	0	0

Initial studies have shown as follows: Inorganic phosphate (as K or

Na salts) at pH 7.2 will lyse cells of *Cl. perfringens*. Under the conditions noted in Table I, 0.015 *M* phosphate was optimal. Inorganic phosphate could be replaced by muscle adenylic acid (10–50 γ) and certain samples of desoxyribosenucleic acid. Not all samples of desoxyribosenucleic acid were active. Yeast adenylic acid, adenosine, adenine, and guanylic acid were not active (Table I). Pyrophosphate, sodium citrate, sodium chloride, and sodium borate did not initiate lysis.

Lysis in the presence of inorganic phosphate was inhibited completely by glucose or glucosamine (Table II). Sodium pyruvate and cysteine also inhibited lysis (Table II).

TABLE II

Inhibition of Lysis of Washed Cells of Clostridium perfringens

Suspensions contained 17 mg. cells (dry weight)/ml. Incubation at 45°C. for 70 min. Lysis measured in a turbidimeter. The pH was 7.2 in all cases.

Suspending medium	Lysis	Inhibition of lysis
	per cent	*per cent*
Water	0	—
Phosphate 0.015 *M*	69	—
Phosphate+glucose 0.015 *M*	5	93
Phosphate+glucose 0.030 *M*	0	100
Phosphate+sodium pyruvate 0.050 *M*	20	71
Phosphate+sodium pyruvate 0.075 *M*	8	89
Phosphate+cysteine-HCl 0.01 *M*	8	89
Phosphate+cysteine-HCl 0.02 *M*	0	100
Phosphate+glucosamine 0.015 *M*	7	90

The lytic property of the cells was apparently independent of the age of the culture from which the cells were obtained.

Toennies and Gallant (1) have recently described a similar lytic phenomenon in lysine-deficient *Streptococcus faecalis*. Studies in this laboratory are being continued to determine the possible mechanism of lysis of *Clostridium perfringens* cells.

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